Aspergillus ochraceus 11 alpha hydroxylase and oxidoreductase

Priority

The present application claims priority under Title 35, United States Code, § 119 of United States Provisional Application Serial No. 60/244,300, filed October 30, 2000.

Field of the invention

The present invention relates to a novel cytochrome P450-like enzyme (*Aspergillus ochraceus* 11 alpha hydroxylase) and an oxidoreductase (*Aspergillus ochraceus* oxidoreductase) isolated from cDNA library generated from the mRNA of *Aspergillus ochraceus* spores. When the cDNA encoding the 11 alpha hydroxylase was co-expressed in *Spodoptera frugiperda* (Sf-9) insect cells with the cDNA encoding human oxidoreductase as an electron donor, it successfully catalyzed the conversion of the steroid substrate 4-androstene-3,17-dione (AD) to 11 alpha-hydroxy-AD as determined by HPLC analysis. The invention also relates to nucleic acid molecules associated with or derived from these cDNAs including complements, homologues and fragments thereof, and methods of using these nucleic acid molecules, to generate, for example, polypeptides and fragments thereof. The invention also relates to the generation of antibodies that recognize the *A. ochraceus* 11 alpha hydroxylase and oxidoreductase and methods of using these antibodies to detect the presence of these native and recombinant polypeptides within unmodified and transformed host cells, respectively. The invention also provides methods of expressing the *Aspergillus* 11 alpha hydroxylase gene separately, or in combination with human or *Aspergillus* oxidoreductase, in heterologous host cells, to facilitate the bioconversion of steroid substrates to their 11 alpha hydroxy-counterparts.

Background of the invention

Microbial transformation or bioconversion reactions have long been used to facilitate the chemical synthesis of a wide variety of pharmaceutical products. Stereospecific reactions carried out under mild enzymatic conditions frequently offer advantages over comparable chemical processes which result in undesireable

side products. Microorganisms also have the ability to carry out simultaneous independent or sequential reactions on a substrate molecule, minimizing the number of distinct steps in a synthesis and reducing the total cost of the desired intermediate or end product.

General features of microbial systems used as biocatalysts for the transformation of organic compounds has been reviewed (See e.g., Goodhue, Charles T., *Microb. Transform. Bioact. Compd.*, 1: 9-44, 1982). Biotransformations can be carried out, for example, in continuous cultures or in batch cultures. Enzymes secreted from the microorganism react with a substrate, and the product can be recovered from the medium. Intracellular enzymes can also react with a substrate if it is able to enter cells by an active or a passive diffusion process. Immobilized, dried, permeabilized, and resting cells, and spores have also been used for microbial transformations. The use of cell extracts and purified enzymes in solution, or immobilized on carriers, may eventually offer significant cost or control advantages over traditional fermentation methods.

Bioconversion reactions have been widely used in the field of steroids (Kieslich, K.; Sebek, O. K. *Annu. Rep. Ferment. Processes* 3: 275-304, 1979; Kieslich, Klaus. *Econ. Microbiol.*, 5 (Microb. Enzymes Bioconvers.): 369-465, 1980). A variety of reactions have been characterized, including hydroxylation, epoxidation, oxidation, dehydrogenation, ring and side chain degradation, reduction, hydrolysis, and isomerization reactions. Many types of microorganisms have also been used including species as diverse, for example, as *Acremonium, Aspergillus, Rhizopus, Fusarium, Penicillium, Streptomyces, Actinomyces, Nocardia, Pseudomonas, Mycobacterium, Arthrobacter* and *Bacillus*.

A variety of approaches have been used to facilitate the hydroxylation of intermediates used in the synthesis of commercially-important steroid compounds. US patent 4,588,683, for example, describes a method of preparing 11 beta, 17 alpha, 20, 21 tetrahydroxy steroids by incubating substrate compounds in a medium comprising a fungal culture of the genus *Curvularia* capable of effecting 11 beta hydroxylation. *Aspergillus ochraceus* cultures and preparations of mycelia have also been used to convert progesterone and other steroids to their corresponding 11 alpha hydroxy forms (Tan, L. and Falardeau, P., 1970; Tan L., and Falardeau P., *J. Steroid Biochem.* **1**: 221-227, 1970; Samanta, T.B. et al., *Biochem. J.* **176**, 593-594, 1978; Jayanthi, C.R. et al., *Biochem. Biophys. Res. Commun.* **106**: 1262-1268, 1982).

The advent of new and expanded clinical uses of steroids for the treatment of a wide variety of disorders has created a need for improved methods for the production of steroid compounds and their intermediates on a commercial scale. U.S. patent 4,559,332, for example, describes a number of methods for the preparation of 20-spiroxane series of steroid compounds, including methods for the preparation of eplerenone methyl hydrogen 9,11α-epoxy-17α-hydroxy-3-oxopregn-4-ene-7α,21-dicarboxylate, γ-lactone (also referred to as eplerenone or epoxymexrenone) and related compounds. WO 98/25948 and U.S. application 09/319,673 describe novel processes for the preparation of 9,11-epoxy steroid compounds, especially those of the 20-spiroxane series and their analogs, novel intermediates useful in the preparation of steroid compounds, and processes for the preparation of such novel intermediates. United States Patent 6,046,023 discloses improved methods for the microbial transformation of canrenone or estr-4-ene-3,17-dione into its 11 α-hydroxy analogue using microorganisms of the genus *Aspergillus, Rhizopus,* and *Pestelotia*, using steroid substrates having a purity of less than 97% and more than 90% at a concentration greater than 10 g/L.

Many modern, systematic approaches needed to optimize bioconversion of particular steroid intermediates are often hindered by insufficient biochemical knowledge of the enzymes involved in their synthesis and degradation. Eukaryotic cytochromes P450 appear to be associated with the endoplasmic reticulum (ER) or mitochondrial membranes. The electron donor for ER-associated cytochrome P450 enzymes is often an FAD/FMN-dependent NADPH-cytochrome P450 oxidoreductase. Electron transfer in the mitochondrial cytochromes P450 is usually mediated by an NADPH-ferredoxin oxidoreductase and ferrodoxin. The specific electron donors known to be involved in mammalian steroidogenesis, are also called adrenodoxin reductase and adrenodoxin, respectively.

While fungal biotransformations are known to be mediated by cytochrome P450 enzymes, many of these enzymes are extremely difficult to purify in an enzymatically-active form (van den Brink et al., *Fungal Genetics and Biology* **23**, 1-17, 1998). Many fungal P450 enzymes appear to be associated with the endoplasmic reticulum (van den Brink et al., *Fungal Genetics and Biology* **23**, 1-17, 1998). Yeast have an adrenodoxin reductase homologue which was shown to couple with a mammalian 11 beta hydroxylase *in vitro.* (Lacour et al., *Journal of Biological Chemistry* **273**, 23984-23992, 1998). In contrast, the electron donor which couples with *Aspergillus ochraceus* 11 alpha hydroxylase was predicted to be an NADPH-cytochrome P450 oxidoreductase (Samanta and Ghosh, *J Steroid Biochem* **28**, 327-32, 1987). The steroid 11 alpha hydroxylation complex in

Rhizopus nigricans also appears to require an NADPH-cytochrome p450 oxidoreductase (Makovec and Breskvar, *Arch Biochem Biophys.* **357**, 310-6, 1998). Amplification of cytochrome *R. nigricans* P450 and NADPH-cytochrome P450 reductase activities in preparations of progesterone-induced fungal mycelia may the facilitate biochemical characterization of both enzymes (Makovec and Breskvar, *Pflugers Arch - Eur J. Physiol* 439(Suppl): R111-R112, 2000).

Aspergillus ochraceus spores have been shown to catalyze the 11 alpha hydroxylation of steroid substrates such as progesterone (Dutta TK, Datta J, Samanta TB, *Biochem. Biophys. Res. Commun.* **192**:119-123, 1993). *A. fumigatus* is also known to exhibit a steroid 11 alpha hydroxylase activity (Smith et al., *J Steroid Biochem Mol Biol* **49**: 93-100, 1994). The *A. fumigatus* enzyme is distinguished from the *A. ochraceus* enzyme, in that it appears to be a cytochrome P450 with dual site-specificity for 11 alpha and 15 beta hydroxylation and, unlike the *A. ochraceus* hydroxylase, appears to be non-inducible.

Despite recent advances in sequencing technologies, detailed knowledge about the structural relationships of fungal cytochrome P450s gleaned from nucleotide sequence data remains primitive. Breskvar *et al.,* (*Biochem. Biophys. Res. Commun* 1991; **178**, 1078-1083, 1991) have described a genomic DNA sequence from *Rhizopus nigricans* for a putative P-450 encoding an 11α–hydroxylase for progesterone. This sequence may not be complete, however, since the predicted amino acid sequence lacks the canonical heme-binding motif, FxxGxxxCxG, which is common to almost all known cytochrome P-450 enzymes. (Nelson *et al*, *Pharmacogenetics* **6**: 1-42, 1996).

The cloning and characterization of the NADPH cytochrome P450 oxidoreductase (cprA) gene of *Aspergillus niger* has been described (van den Brink, J., et al., Genbank accession numbers Z26938, CAA81550, 1993, unpublished). The primary structure of *Saccharomyces cerevisiae* NADPH-cytochrome P450 reductase has also been deduced from the nucleotide sequence of its cloned gene (Yabusaki et al., *J. Biochem.* **103**(6): 1004-1010, 1988).

Several other approaches have been used to facilitate the cloning and analysis of steroid enzymes. U.S. patents 5,422,262, 5,679,521, and European patent EP 0 528 906 B1, for example, describes the expression cloning of steroid 5 alpha reductase, type 2. U.S. patent 5,869,283, for example, describes an expression cassette comprising heterologous DNAs encoding two or more enzymes, each catalyzing an oxidation step involved conversion of cholesterol into

hydrocortisone, including the conversion of cholesterol to pregnenolone; the conversion of pregnenolone to progesterone; the conversion of progesterone to 17 α-hydroxy-progesterone; the conversion of 17 α-hydroxyprogesterone to cortexolone; and the conversion of cortexolone to hydrocortisone.

The sequences of *Aspergillus ochraceus* 11 alpha hydroxylase and *A. ochraceus* oxidoreductase have not been reported. Knowledge about their sequence could greatly facilitate the development of expression vectors and recombinant host strains that can carry out more efficient bioconversions of steroid intermediates and the synthesis of end products on a commercial scale without the problems associated with partially-characterized host strains or an incomplete understanding of the enzymes involved in steroidogenesis. The present invention overcomes many of the limitations discussed above by identifying enzymes capable of carrying out the 11 alpha hydroxylation of steroids. This approach not only greatly facilitates the use of 11 alpha hydroxylation, but also permits the development of new strategies for the identification of similar enzymes from other fungi, the cloning of other enzymes involved in steroidogenesis from *Aspergillus ochraceus* and other microorganisms, and the development of improved host strains or methods using free cells or immobilized cells or enzymes in bioconversion reactions. Similar approaches could also be developed to aid in the construction of expression vectors and recombinant host strains that are more amenable to propagation and control than wild-type microorganisms now commonly used for bioconversion in large scale bioreactors.

Summary of the invention

In its broadest scope, the present invention provides a method to clone enzymes involved in steroid metabolism and use of these enzymes to produce novel steroid intermediates and end-products. One aspect of the claimed invention is to provide a novel enzyme 11 alpha hydroxylase and oxidoreductase, and their nucleic acids, proteins, peptides, fragments, and homologues. The invention also relates to methods of identifying and cloning other enzymes involved in steroid metabolism. The invention also covers novel vectors and host cells, a novel method for making heterologous proteins by using the above vectors, and a method for identifying the substrate specificity of the cloned enzymes.

The invention provides a means for determining the substrate specificity of the cloned 11 alpha hydroxylase, allelic variants, muteins, and fusion proteins thereof, permitting evaluation of a broad array of steroid substrates including 3

keto delta 4,5 steroids (3 keto delta 4 steroids); 3 keto delta 4, 5 delta 6, 7 steroids (3 keto delta 4 delta 6 steroids); 3 keto delta 6, 7 steroids (3 keto delta 6 steroids); or 3 keto delta 1, 2 delta 4, 5 steroids (3 keto delta 1 delta 4 steroids). Preferred substrates for testing include (a) canrenone; (b) androstenedione; (c) aldona; (d) ADD (1,4 androstenedienedione) (e) mexrenone; (f) 6 beta mexrenone; (g) 9 alpha mexrenone; (h) 12 beta mexrenone; (i) delta 12 mexrenone; (j) testosterone; (k) progesterone; (l) mexrenone 6,7-bis-lactone; and (m) mexrenone 7,9-bislactone. Preferably the cloned 11 alpha hydroxylase, allelic variants, muteins, and fusion proteins thereof do not also catalyze a second hydroxylation selected from the group consisting of 15 alpha or beta hydroxylation, 6 alpha or beta hydroxylation, 7 alpha or beta hydroxylation, 9 alpha or beta hydroxylation, 12 alpha or beta hydroxylation, and 17 alpha or beta hydroxylation of substrates selected from the group consisting of 3 keto delta 4,5 steroids; 3 keto delta 4, 5 delta 6, 7 steroids; or 3 keto delta 6, 7 steroids. Most preferably the cloned 11 alpha hydroxylase, allelic variants, muteins, and fusion proteins thereof do not catalyze the 15 beta hydroxylation of substrates selected from the group consisting of 3 keto delta 4,5 steroids; 3 keto delta 4, 5 delta 6, 7 steroids; or 3 keto delta 6, 7 steroids.

The invention provides an isolated and purified nucleic acid, encoding *Aspergillus ochraceus* 11 alpha hydroxylase. It also provides an isolated DNA, cDNA, gene, and an allele of the gene encoding *Aspergillus ochraceus* 11 alpha hydroxylase. Preferably the isolated and purified nucleic acid is as set forth in SEQ ID NO: 01. Preferably the isolated DNA, cDNA, gene, and an allele of the gene is as set forth in SEQ ID NO: 01.

The invention provides an isolated protein having the amino acid sequence of *Aspergillus ochraceus* 11 alpha hydroxylase. It also provides an isolated variant of *Aspergillus ochraceus* 11 alpha hydroxylase, and a fusion protein comprising this hydroxylase. Preferably the protein is as set forth in SEQ ID NO: 2. It also provides for variant of the protein set forth in SEQ ID NO: 2.; a polypeptide which comprises SEQ ID NO: 2 with at least one conservative amino acid substitution; polypeptides, with an amino acid sequence at least 99%, 95%, 90%, 75%, and 50% identical to SEQ ID NO: 2.

The invention provides an isolated and purified nucleic acid, encoding *Aspergillus ochraceus* 11 alpha oxidoreductase. It also provides an isolated DNA, cDNA, gene, and allele of the gene encoding *Aspergillus ochraceus* oxidoreductase. Preferably, the isolated and purified nucleic acid, wherein said nucleic acid

sequence is as set forth in SEQ ID NO: 5. It also provides for an isolated DNA, cDNA, gene, and allele of the gene set forth in SEQ ID NO: 5.

The invention provides an isolated protein having the amino acid sequence of *Aspergillus ochraceus* oxidoreductase. It also provides an isolated variant of the protein having the amino acid sequence of *Aspergillus ochraceus* oxidoreductase, and a fusion protein comprising the amino acid sequence of *Aspergillus ochraceus* oxidoreductase. Preferably the isolated protein has the amino acid sequence set forth in SEQ ID NO: 6. It also provides an isolated variant of a protein set forth in SEQ ID NO: 6. a purified polypeptide, the amino acid sequence of which comprises SEQ ID NO: 6 with at least one conservative amino acid substitution; and a polypeptides with an amino acid sequence at least 99%, 95%, 90%, 75%, and 50% identical to SEQ ID NO: 6.

The invention provides an isolated and purified nucleic acid encoding an enzyme that can catalyze the 11 alpha hydroxylation of 3 keto delta 4,5 steroids (3 keto delta 4 steroids); 3 keto delta 4, 5 delta 6, 7 steroids (3 keto delta 4 delta 6 steroids); 3 keto delta 6, 7 steroids (3 keto delta 6 steroids); or 3 keto delta 1, 2 delta 4, 5 steroids (3 keto delta 1 delta 4 steroids). Preferably the enzyme does not catalyze the 15 beta hydroxylation of 3 keto delta 4,5 steroids; 3 keto delta 4, 5 delta 6, 7 steroids; or 3 keto delta 6, 7 steroids. More preferably, the hydroxylation is selected from the group consisting of: (a) canrenone to 11 alpha hydroxy canrenone; (b) androstenedione to 11 alpha hydroxy androstenedione; (c) aldona to 11 alpha hydroxy aldona; (d) ADD (1,4 androstenedienedione) to 11 alpha hydroxy ADD; (e) mexrenone to 11 alpha hydroxy mexrenone; (f) 6 beta mexrenone to 11 alpha hydroxy 6 beta mexrenone; (g) 9 alpha mexrenone to 11 alpha hydroxy 9 alpha mexrenone; (h) 12 beta mexrenone to 11 alpha hydroxy 12 beta mexrenone; (i) delta 12 mexrenone to 11 alpha hydroxy delta 12 mexrenone; (j) testosterone to 11 alpha hydroxy testosterone; (k) progesterone to 11 alpha hydroxy progesterone; (l) mexrenone 6,7-bis-lactone to 11 alpha hydroxy mexrenone 6,7-bis-lactone; and (m) mexrenone 7,9-bislactone to 11 alpha hydroxy mexrenone 7,9-bislactone. More preferably, the hydroxylation is selected from the group consisting of: (a) canrenone to 11 alpha hydroxy canrenone; (b) androstenedione to 11 alpha hydroxy androstenedione; (c) aldona to 11 alpha hydroxy aldona; and (d) ADD (1,4 androstenedienedione) to 11 alpha hydroxy ADD. Most preferably the hydroxylation is from canrenone to 11 alpha hydroxy canrenone.

The invention also provides a method of expressing a protein that can catalyze the 11 alpha hydroxylation of 3 keto delta 4,5 steroids; 3 keto delta 4, 5

delta 6, 7 steroids; 3 keto delta 6, 7 steroids; or 3 keto delta 1, 2 delta 4, 5 steroids comprising; (a) transforming or transfecting host cells with an expression cassette comprising a promoter operably linked to a nucleic acid that encodes said protein, and (b) expressing said protein in said host cells. The invention also provides for a method of producing the protein further comprising the step of recovering said protein. Preferably, this protein is *Aspergillus ochraceus* 11 alpha hydroxylase. More preferably, this method further comprises expressing an electron donor protein, wherein said electron donor protein can donate electrons to said protein that can catalyze the 11 alpha hydroxylation of 3 keto delta 4,5 steroids; 3 keto delta 4, 5 delta 6, 7 steroids; 3 keto delta 6, 7 steroids; or 3 keto delta 1, 2 delta 4, 5 steroids. Preferably, the electron donor protein is selected from the group consisting of human oxidoreductase and *Aspergillus ochraceus* oxidoreductase. More preferably the electron donor protein is *Aspergillus ochraceus* oxidoreductase. More preferably, the nucleic acid encoding said steroid 11 alpha hydroxylase and said electron donor protein are on separate expression cassettes. More preferably, the nucleic acid encoding said steroid 11 alpha hydroxylase and said electron donor protein are on the same expression cassettes. Even more preferably, the steroid 11 alpha hydroxylase is *Aspergillus ochraceus* 11 alpha hydroxylase and said electron donor protein is human oxidoreductase. Even more preferably, the steroid 11 alpha hydroxylase is *Aspergillus ochraceus* 11 alpha hydroxylase and said electron donor protein is *Aspergillus ochraceus* oxidoreductase. Preferably, the expression cassette is on an expression vector. More preferably, the expression vector is a baculovirus. Even more preferably, the baculovirus is a nuclear polyhedrosis virus is selected from the group consisting of *Autographa californica* nuclear polyhedrosis virus and *Bombyx mori* nuclear polyhedrosis virus. Most preferably, the nuclear polyhedrosis virus is *Autographa californica* nuclear polyhedrosis virus. Preferably, the host cells are insect cells. More preferably, the insect cells are selected from the group consisting of *Spodoptera frugiperda, Trichoplusia ni, Autographa californica,* and *Manduca sexta* cells. Most preferably the insect cells are *Spodoptera frugiperda* cells. The invention also provides a for a method of expressing a protein wherein the *Aspergillus ochraceus* 11 alpha hydroxylase is SEQ ID NO: 2; the human oxidoreductase is SEQ ID NO: 4; and the *Aspergillus ochraceus* oxidoreductase is SEQ ID NO: 6.

The invention also provides for an isolated and purified polypeptide that can catalyze the 11 alpha hydroxylation of 3 keto delta 4,5 steroids (3 keto delta 4 steroids); 3 keto delta 4, 5 delta 6, 7 steroids (3 keto delta 4 delta 6 steroids); 3 keto delta 6, 7 steroids (3 keto delta 6 steroids); or 3 keto delta 1, 2 delta 4, 5 steroids (3

keto delta 1 delta 4 steroids). Preferably, the polypeptide does not catalyze the 15 beta hydroxylation of 3 keto delta 4,5 steroids; 3 keto delta 4, 5 delta 6, 7 steroids; or 3 keto delta 6, 7 steroids. More preferably, the hydroxylation is selected from the group consisting of: (a) canrenone to 11 alpha hydroxy canrenone; (b) androstenedione to 11 alpha hydroxy androstenedione; (c) aldona to 11 alpha hydroxy aldona; (d) ADD (1,4 androstenedienedione) to 11 alpha hydroxy ADD; (e) mexrenone to 11 alpha hydroxy mexrenone; (f) 6 beta mexrenone to 11 alpha hydroxy 6 beta mexrenone; (g) 9 alpha mexrenone to 11 alpha hydroxy 9 alpha mexrenone; (h) 12 beta mexrenone to 11 alpha hydroxy 12 beta mexrenone; (i) delta 12 mexrenone to 11 alpha hydroxy delta 12 mexrenone; (j) testosterone to 11 alpha hydroxy testosterone; (k) progesterone to 11 alpha hydroxy progesterone; (l) mexrenone 6,7-bis-lactone to 11 alpha hydroxy mexrenone 6,7-bis-lactone; and (m) mexrenone 7,9-bislactone to 11 alpha hydroxy mexrenone 7,9-bislactone. More preferably, the hydroxylation is selected from the group consisting of: (a) canrenone to 11 alpha hydroxy canrenone; (b) androstenedione to 11 alpha hydroxy androstenedione; (c) aldona to 11 alpha hydroxy aldona; and (d) ADD (1,4 androstenedienedione) to 11 alpha hydroxy ADD. Most preferably the hydroxylation is from canrenone to 11 alpha hydroxy canrenone.

The invention also provides for an expression cassette comprising a promoter operably linked to an isolated and purified nucleic acid encoding a polypeptide that can catalyze the 11 alpha hydroxylation of 3 keto delta 4,5 steroids (3 keto delta 4 steroids); 3 keto delta 4, 5 delta 6, 7 steroids (3 keto delta 4 delta 6 steroids); 3 keto delta 6, 7 steroids (3 keto delta 6 steroids); or 3 keto delta 1, 2 delta 4, 5 steroids (3 keto delta 1 delta 4 steroids). More preferably, the hydroxylation is selected from the group consisting of: (a) canrenone to 11 alpha hydroxy canrenone; (b) androstenedione to 11 alpha hydroxy androstenedione; (c) aldona to 11 alpha hydroxy aldona; (d) ADD (1,4 androstenedienedione) to 11 alpha hydroxy ADD; (e) mexrenone to 11 alpha hydroxy mexrenone; (f) 6 beta mexrenone to 11 alpha hydroxy 6 beta mexrenone; (g) 9 alpha mexrenone to 11 alpha hydroxy 9 alpha mexrenone; (h) 12 beta mexrenone to 11 alpha hydroxy 12 beta mexrenone; (i) delta 12 mexrenone to 11 alpha hydroxy delta 12 mexrenone; (j) testosterone to 11 alpha hydroxy testosterone; (k) progesterone to 11 alpha hydroxy progesterone; (l) mexrenone 6,7-bis-lactone to 11 alpha hydroxy mexrenone 6,7-bis-lactone; and (m) mexrenone 7,9-bislactone to 11 alpha hydroxy mexrenone 7,9-bislactone. More preferably, the hydroxylation is selected from the group consisting of: (a) canrenone to 11 alpha hydroxy canrenone; (b) androstenedione to 11 alpha hydroxy androstenedione; (c) aldona to 11 alpha

hydroxy aldona; and (d) ADD (1,4 androstenedienedione) to 11 alpha hydroxy ADD. Most preferably the hydroxylation is from canrenone to 11 alpha hydroxy canrenone.

The invention also provides for an expression cassette comprising a promoter operably linked to an isolated and purified nucleic acid encoding *Aspergillus ochraceus* oxidoreductase. Preferably the nucleic acid is SEQ ID NO: 6.

The invention also provides for an expression cassette comprising a heterologous DNA encoding an enzyme from the metabolic pathway for the synthesis of sitosterol to eplerenone wherein said enzyme catalyzes at least one conversion selected from the group consisting of: (a) canrenone to 11 alpha hydroxy canrenone; (b) androstenedione to 11 alpha hydroxy androstenedione; (c) aldona to 11 alpha hydroxy aldona; (d) ADD (1,4 androstenedienedione) to 11 alpha hydroxy ADD; (e) mexrenone to 11 alpha hydroxy mexrenone; (f) 6 beta mexrenone to 11 alpha hydroxy 6 beta mexrenone; (g) 9 alpha mexrenone to 11 alpha hydroxy 9 alpha mexrenone; (h) 12 beta mexrenone to 11 alpha hydroxy 12 beta mexrenone; (i) delta 12 mexrenone to 11 alpha hydroxy delta 12 mexrenone; (j) testosterone to 11 alpha hydroxy testosterone; and (k) progesterone to 11 alpha hydroxy progesterone; (l) mexrenone 6,7-bis-lactone to 11 alpha hydroxy mexrenone 6,7-bis-lactone; and (m) mexrenone 7,9-bislactone to 11 alpha hydroxy mexrenone 7,9-bislactone and wherein the heterologous DNA is operably linked to control sequences required to express the encoded enzymes in a recombinant host. Preferably the heterologous DNA coding sequences in the expression cassette are selected from the group consisting of the following genus and species: *Aspergillus ochraceus*, *Aspergillus ochraceus*, *Aspergillus niger*, *Aspergillus nidulans*, *Rhizopus oryzae*, *Rhizopus stolonifer*, *Streptomyces fradiae*, *Bacillus megaterium*, *Pseudomonas cruciviae*, *Trichothecium roseum*, *Fusarium oxysporum Rhizopus arrhizus*, *Absidia coerula*, *Absidia glauca*, *Actinomucor elegans*, *Aspergillus flavipes*, *Aspergillus fumigatus*, *Beauveria bassiana*, *Botryosphaeria obtusa*, *Calonectria decora*, *Chaetomium cochliodes*, *Corynespora cassiicola*, *Cunninghamella blakesleeana*, *Cunninghamella echinulata*, *Cunninghamella elegans*, *Curvularia clavata*, *Curvularia lunata*, *Cylindrocarpon radicicola*, *Epicoccum humicola*, *Gongronella butleri*, *Hypomyces chrysospermus*, *Monosporium olivaceum*, *Mortierella isabellina*, *Mucor mucedo*, *Mucor griseocyanus*, *Myrothecium verrucaria*, *Nocardia corallina*, *Paecilomyces carneus*, *Penicillum patulum*, *Pithomyces atroolivaceus*, *Pithomyces cynodontis*, *Pycnosporium* sp., *Saccharopolyspora erythrae*, *Sepedonium chrysospermum*, *Stachylidium bicolor*, *Streptomyces hyqroscopicus*, *Streptomyces purpurascens*,

Syncephalastrum racemosum, *Thamnostylum piriforme*, *Thielavia terricola*, and *Verticillium theobromae, Cephalosporium aphidicola*, *Cochliobolus lunatas*, *Tieghemella orchidis*, *Tieghemella hyalospora*, *Monosporium olivaceum*, *Aspergillus ustus*, *Fusarium graminearum*, *Verticillium glaucum*, and *Rhizopus nigricans*. More preferably, the genus and species are selected from the group consisting of *Aspergillus ochraceus*, *Aspergillus ochraceus*, *Aspergillus niger*, *Aspergillus nidulans*, *Rhizopus oryzae*, *Rhizopus stolonifer*, *Streptomyces fradiae*, *Bacillus megaterium*, *Pseudomonas cruciviae*, *Trichothecium roseum*, *Fusarium oxysporum, Rhizopus arrhizus, and Monosporium olivaceum*. Most preferably, genus and species is *Aspergillus ochraceus*.

Preferably, the recombinant host cell and progeny thereof comprise at least one expression cassette. More preferably, the host is a microorganism. Most preferably, the host is a bacterium. The invention also provides for a process for making one or more enzymes from the metabolic pathway for the transformation of sitosterol to eplerenone comprising incubating the recombinant host cell in a nutrient medium under conditions where the one or more enzymes encoded by the heterologous DNA are expressed and accumulate. More preferably the process comprises the steps of: (a) incubating the compound to be oxidized in the presence the recombinant host cells under conditions where the compound is hydroxylated and the hydroxylated product accumulates, and (b) recovering the hydroxylated product. Most preferably, the process comprises the steps of: (a) incubating the compound to be oxidized in the presence of the enzymes produced under conditions where the compound is hydroxylated and the hydroxylated product accumulates, and (b) recovering the hydroxylated product. The invention also provides for a host cells harboring an expression cassette. More preferably the expression cassette is integrated into the chromosome of said host cell. More preferably, the expression cassette is integrated into an expression vector.

The invention also provides for a method of determining the specific activity of a cloned 11 alpha hydroxylase comprising the steps of; (a) transforming host cells with an expression vector comprising a nucleic acid that encodes said 11 alpha hydroxylase, (b) expressing said 11 alpha hydroxylase in said host cells; (c) preparing subcellular membrane fractions from said cells, (d) incubating said subcellular membrane fractions with a steroid substrate, and (e) monitoring conversion of the steroid substrate to its 11 alpha hydroxy steroid counterpart. Preferably, the further comprises transforming host cells with an expression vector nucleic acid that encodes an oxidoreductase, and expressing said oxidoreductase in said host cells. More preferably, the oxidoreductase is human or *Aspergillus*

ochraceus. Most preferably the oxidoreductase is human oxidoreductase. Most preferably the oxidoreductase is *Aspergillus ochraceus* oxidoreductase.

The invention also provides for a protein having SEQ ID NO: 2 and variants thereof that are at least 95% identical to SEQ ID NO: 2 and catalyze the 11 alpha hydroxylation of 3 keto delta 4,5 steroids; 3 keto delta 4, 5 delta 6, 7 steroids; 3 keto delta 6, 7 steroids; or 3 keto delta 1, 2 delta 4, 5 steroids, wherein said hydroxylation is selected from the group consisting of: (a) canrenone to 11 alpha hydroxy canrenone; (b) androstenedione to 11 alpha hydroxy androstenedione; (c) aldona to 11 alpha hydroxy aldona; (d) ADD (1,4 androstenedienedione) to 11 alpha hydroxy ADD; (e) mexrenone to 11 alpha hydroxy mexrenone; (f) 6 beta mexrenone to 11 alpha hydroxy 6 beta mexrenone; (g) 9 alpha mexrenone to 11 alpha hydroxy 9 alpha mexrenone; (h) 12 beta mexrenone to 11 alpha hydroxy 12 beta mexrenone; (i) delta 12 mexrenone to 11 alpha hydroxy delta 12 mexrenone; (j) testosterone to 11 alpha hydroxy testosterone; and (k) progesterone to 11 alpha hydroxy progesterone. Preferably the enzyme does not catalyze the 15 beta hydroxylation of 3 keto delta 4,5 steroids; 3 keto delta 4, 5 delta 6, 7 steroids; or 3 keto delta 6, 7 steroids.

The invention provides an isolated and purified nucleic acid encoding an enzyme that can catalyze the 11 alpha hydroxylation of 3 keto delta 4,5 steroids (3 keto delta 4 steroids); 3 keto delta 4, 5 delta 6, 7 steroids (3 keto delta 4 delta 6 steroids); 3 keto delta 6, 7 steroids (3 keto delta 6 steroids); or 3 keto delta 1, 2 delta 4, 5 steroids (3 keto delta 1 delta 4 steroids) wherein the hydroxylation is selected from the group consisting of: (a) canrenone to 11 alpha hydroxy canrenone; (b) androstenedione to 11 alpha hydroxy androstenedione; (c) aldona to 11 alpha hydroxy aldona; (d) ADD (1,4 androstenedienedione) to 11 alpha hydroxy ADD; (e) mexrenone to 11 alpha hydroxy mexrenone; (f) 6 beta mexrenone to 11 alpha hydroxy 6 beta mexrenone; (g) 9 alpha mexrenone to 11 alpha hydroxy 9 alpha mexrenone; (h) 12 beta mexrenone to 11 alpha hydroxy 12 beta mexrenone; (i) delta 12 mexrenone to 11 alpha hydroxy delta 12 mexrenone; (j) testosterone to 11 alpha hydroxy testosterone; and (k) progesterone to 11 alpha hydroxy progesterone. Preferably the enzyme does not catalyze the 15 beta hydroxylation of 3 keto delta 4,5 steroids; 3 keto delta 4, 5 delta 6, 7 steroids; or 3 keto delta 6, 7 steroids.

The invention also provides for a purified polypeptide, the amino acid sequence of which is selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25.

The invention provides for a purified immunogenic polypeptide, the amino acid sequence of which comprises at least ten consecutive residues of SEQ ID NO: 2.

The invention provides for an isolated and purified antibody having a binding specificity for 11 alpha hydroxylase having an amino acid sequence as shown in SEQ ID NO: 2. Preferably the antibody binds to a protein region selected from the group consisting of (a) the N-terminal amino acids 1-10 of SEQ ID NO: 2; (b) the last 10 C-terminal amino acids of SEQ ID NO: 2; (c) amino acids SEQ ID NO: 23; (d) amino acids SEQ ID NO: 24; and (e) amino acids SEQ ID NO: 25. Preferably the antibody is purified on a peptide column, wherein said peptide is selected from the group consisting of: (a) the N-terminal amino acids 1-10 of SEQ ID NO: 2; (b) the last 10 C-terminal amino acids of SEQ ID NO: 2; (c) amino acids SEQ ID NO: 23; (d) amino acids SEQ ID NO: 24; and (e) amino acids SEQ ID NO: 25.

The invention also provides for a purified polypeptide, the amino acid sequence of which is selected from the group consisting of SEQ ID NO: 26.

The invention also provides for a purified immunogenic polypeptide, the amino acid sequence of which comprises at least ten consecutive residues of SEQ ID NO: 6.

The invention also provides for an isolated and purified antibody having a binding specificity for 11 alpha hydroxylase having an amino acid sequence as shown in SEQ ID NO: 6. Preferably the antibody binds to a protein region selected from the group consisting of (a) the N-terminal amino acids 1-10 of SEQ ID NO: 6; (b) the last 10 C-terminal amino acids of SEQ ID NO: 6; and (c) amino acids SEQ ID NO: 26. More preferably, the antibody is purified on a peptide column, wherein said peptide is selected from the group consisting of: (a) the N-terminal amino acids 1-10 of SEQ ID NO: 6; (b) the last 10 C-terminal amino acids of SEQ ID NO: 6; and (c) amino acids SEQ ID NO: 26.

The invention also provides for a composition comprising an antibody described above in an effective carrier, vehicle, or auxiliary agent. It also provides for a composition comprising such an antibody and a solution. The antibody may be a polyclonal antibody. The antibody may also be a monoclonal antibody. The antibody may be conjugated to an immunoaffinity matrix. The invention also provides for a method of using an immunoaffinity matrix to purify a polypeptide from a biological fluid or cell lysate. Preferably the immunoaffinity matrix is

SEPHAROSE 4B. More preferably the method of using an immunoaffinity matrix to purify a polypeptide from a biological fluid or cell lysate uses SEPHAROSE 4B as an immunoaffinity matrix. More preferably, the method of using an immunoaffinity matrix to purify a polypeptide from a biological fluid or cell lysate. uses SEPHAROSE 4B as an immunoaffinity matrix.

The invention also provides for a method of using a peptide column to purify an antibody, wherein said peptide is selected from the group consisting of: (a) the N-terminal amino acids 1-10 of SEQ ID NO: 2; (b) the last 10 C-terminal amino acids of SEQ ID NO: 2; (c) amino acids SEQ ID NO: 23; (d) amino acids SEQ ID NO: 24; and (e) amino acids SEQ ID NO: 25.

The invention also provides for a method of using a peptide column to purify an antibody, wherein said peptide is selected from the group consisting of: (a) the N-terminal amino acids 1-10 of SEQ ID NO: 6; (b) the last 10 C-terminal amino acids of SEQ ID NO: 6; and (c) amino acids SEQ ID NO: 26.

The invention also provides for a method of detecting a first polypeptide in a biological fluid, wherein said first polypeptide is selected from the group consisting of 11 alpha hydroxylase and oxidoreductase, comprising the following steps: (a) contacting said fluid with a second polypeptide, having a binding specificity for said first polypeptide, and (b) assaying the presence of said second polypeptide to determine the level of said first polypeptide. Preferably, the second polypeptide is an antibody. More preferably, the second polypeptide is radiolabeled.

The invention also provides for a process for producing an isolated nucleic acid comprising hybridizing SEQ ID NO: 1 to genomic DNA in 6XSSC and 65°C and isolating the nucleic acid detected with SEQ ID NO: 1. The invention also provides for an isolated DNA nucleic acid prepared according to this process.

The invention also provides for an isolated nucleic acid that specifically hybridizes under highly stringent conditions to the complement of the sequence set forth in SEQ ID NO: 1.

The invention also provides for a process for producing an isolated nucleic comprising hybridizing SEQ ID NO: 5 to genomic DNA in 6XSSC and 65°C and isolating the nucleic acid detected with SEQ ID NO: 5. The invention also provides for an isolated DNA nucleic acid prepared according to this process.

The invention also provides for an isolated nucleic acid that specifically hybridizes under highly stringent conditions to the complement of the sequence set forth in SEQ ID NO: 5.

The invention also provides for a DNA construct which alters the expression of a 11 alpha hydroxylase gene not normally expressed in a cell when said DNA construct is inserted into chromosomal DNA of the cell, said DNA construct comprising: (a) a targeting sequence; (b) a regulatory sequence; and (c) the structural gene for a steroid 11 alpha hydroxylase. The invention also provides for a host cell harboring this DNA construct.

The invention also provides for a DNA construct which alters the expression of a 11 alpha hydroxylase gene not normally expressed in a cell when said DNA construct is inserted into chromosomal DNA of the cell, said DNA construct comprising: (a) a targeting sequence; (b) a regulatory sequence; and (c) the structural gene for a steroid oxidoreductase. The invention also provides for a host cell harboring this DNA construct.

The invention also provides for use of a host cell harboring a cloned 11 alpha hydroxylase for the manufacture of a medicament for therapeutic application to treat heart disease, inflammation, arthritis, or cancer.

The invention also provides for a composition comprising from about 0.5-to about 500 g/L molasses, 0.5-50 g/L cornsteep liquid, 0.5-50 g/L KH_2PO_4, 2.5-250 g/L NaCl, 2.5-250 g/L glucose, and 0.04-4 g/L progesterone, pH 3.5-7. Preferably, this composition is comprised of from about 10-250 g/L molasses, 1-25 g/L cornsteep liquid, 1-25 g/L KH_2PO_4, 5-125 g/L NaCl, 5-125 g/L glucose, and 0.08-2 g/L progesterone, pH 4.5-6.5. More preferably, the composition is comprised of from about 25-100 g/L molasses, 2.5-10 g/L cornsteep liquid, 2.5-10 g/L KH_2PO_4, 12.5-50 g/L NaCl, 12.5-50 g/L glucose, and 0.2-0.8 g/L progesterone, pH 5.5-6.0. Most preferably the composition comprises about 50 g/L molasses, 5 g/L cornsteep liquid, 5 g/L KH_2PO_4, 25 g/L NaCl, 25 g/L glucose, 20 g/L agar, and 0.4 g/L progesterone, pH 5.8.

The invention also provides for a semisolid formulation of any of the compositions described above, further comprising from about 4-100 g/L agar. Preferably the agar is at a concentration of from about 10-40 g/L agar. More preferably, the agar is about 20 g/L agar.

The invention also provides for the use of any of the compositions describe above to produce spores from the microorganism selected from the group consisting of *Aspergillus ochraceus*, *Aspergillus niger*, *Aspergillus nidulans*, *Rhizopus oryzae*, *Rhizopus stolonifer*, and *Trichothecium roseum*, *Fusarium oxysporum Rhizopus arrhizus*, *Monosporium olivaceum. Penicillum chrysogenum,* and *Absidia coerula.* Preferably, the composition is used to produce spores from *Aspergillus ochraceus.*

Definitions

The following is a list of abbreviations and the corresponding meanings as used interchangeably herein:

11 alpha hydroxycanrenone = 11 alpha hydroxy-4-androstene-3,17-dione ($C_{22}H_{28}O_4$, MW 356.46)

AcNPV = *Autographa californica* nuclear polyhedrosis virus, a member of the Baculoviridae family of insect viruses

AD = androstenedione or 4-androstene-3,17-dione ($C_{22}H_{28}O_3$, MW 340.46)

aldadiene = canrenone

Amp = ampicillin

attTn7 = attachment site for Tn7 (a preferential site for Tn7 insertion into bacterial chromosomes)

bacmid = recombinant baculovirus shuttle vector isolated from *E. coli*

Bluo-gal = halogenated indolyl-β-D-galactoside

bp = base pair(s)

Cam = chloramphenicol

cDNA = complementary DNA

DMF = N,N-dimethylformamide

ds = double-stranded

eplerenone or epoxymexrenone = methyl hydrogen 9,11α-epoxy-17α-hydroxy-3-oxopregn-4-ene-7α,21-dicarboxylate, γ-lactone (MW 414.5)

g = gram(s)

Gen = gentamicin

hoxr = human oxidoreductase

HPLC = high performance liquid chromatography

hydroxycanrenone = 11 alpha- or 11 beta-hydroxycanrenone

IPTG = isopropyl-β-D-thiogalactopyranoside

Kan = kanamycin

kb = kilobase(s), 1000 bp(s)

mb = megabase(s)

Me = methyl

mg = milligram(s)

ml or mL = milliliter(s)

mm = millimeter

mM = millimolar

NMR = nuclear magnetic resonance

oxr = oxidoreductase

PCR = polymerase chain reaction

r = resistant or resistance

RP-HPLC = reverse phase high performance liquid chromatography

RT = room temperature

RT-PCR = reverse transcriptase polymerase chain reaction

s = sensitive

SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

Spc/Str = spectinomycin/streptomycin

Tet = tetracycline

Tn = transposon

ts = temperature-sensitive

U = units

ug or μg = microgram(s)

ul or μl = microliter(s)

X-gal = 5-bromo-3-chloro-indolyl-β-D-galactopyranoside

X-gluc = 5-bromo-3-chloro-indolyl-β-D-glucopyranoside

The following is a list definitions of various terms used herein:

The species "*Aspergillus ochraceus* NRRL 405" means the filamentous fungus *Aspergillus ochraceus* NRRL 405, accession number 18500, obtained from the American Type Culture Collection (ATCC). *A. ochraceus* NRRL 405 and *A. ochraceus* ATCC 18500 are the same strain, catalogued differently.

The term "amino acid(s)" means all naturally occurring L-amino acids, including norleucine, norvaline, homocysteine, and ornithine.

The term "degenerate" means that two nucleic acid molecules encode for the same amino acid sequences but comprise different nucleotide sequences.

The term "fragment" means a nucleic acid molecule whose sequence is shorter than the target or identified nucleic acid molecule and having the identical,

the substantial complement, or the substantial homologue of at least 10 contiguous nucleotides of the target or identified nucleic acid molecule.

The term "fusion protein" means a protein or fragment thereof that comprises one or more additional peptide regions not derived from that protein.

The term "probe" means an agent that is utilized to determine an attribute or feature (e.g. presence or absence, location, correlation, etc.) of a molecule, cell, tissue, or organism.

The term "promoter" is used in an expansive sense to refer to the regulatory sequence(s) that control mRNA production. Such sequences include RNA polymerase binding sites, enhancers, etc.

The term "protein fragment" means a peptide or polypeptide molecule whose amino acid sequence comprises a subset of the amino acid sequence of that protein.

The term "recombinant" means any agent (e.g., DNA, peptide, etc.), that is, or results from, however indirectly, human manipulation of a nucleic acid molecule.

The term "selectable or screenable marker genes" means genes whose expression can be detected by a probe as a means of identifying or selecting for transformed cells.

The term "specifically bind" means that the binding of an antibody or peptide is not competitively inhibited by the presence of non-related molecules.

The term "specifically hybridizing" means that two nucleic acid molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure.

The term "substantial complement" means that a nucleic acid sequence shares at least 80% sequence identity with the complement.

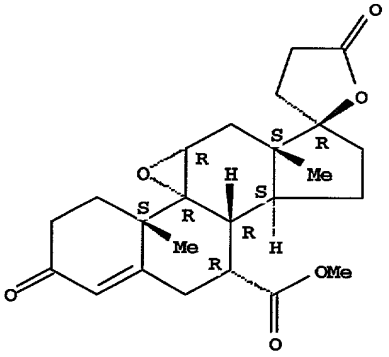
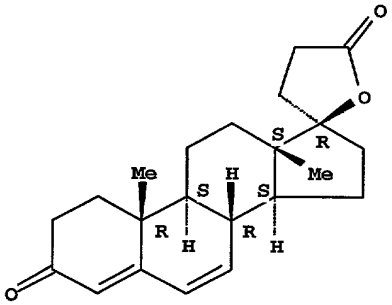
The term "substantial fragment" means a nucleic acid fragment which comprises at least 100 nucleotides.

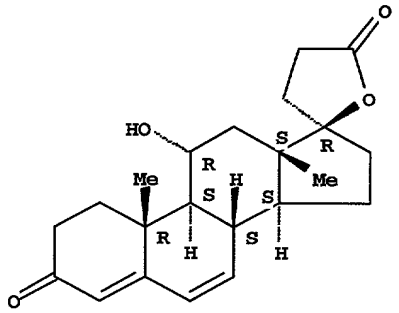
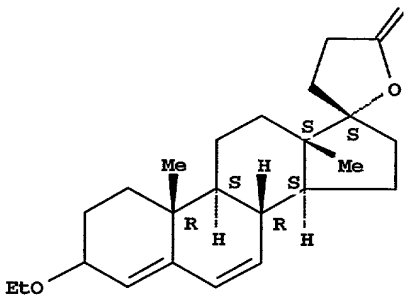
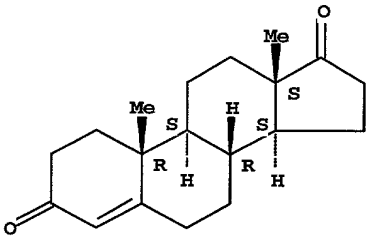
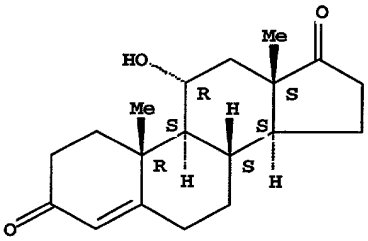
The term "substantial homologue" means that a nucleic acid molecule shares at least 80% sequence identity with another.

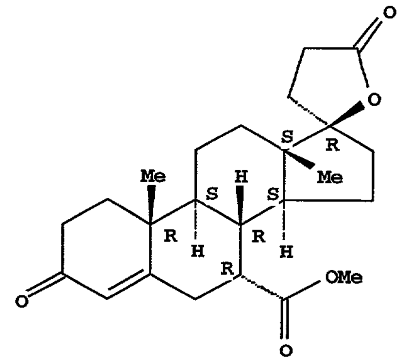
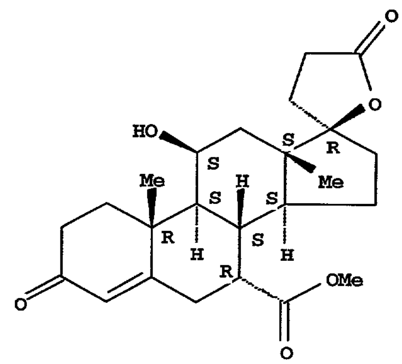
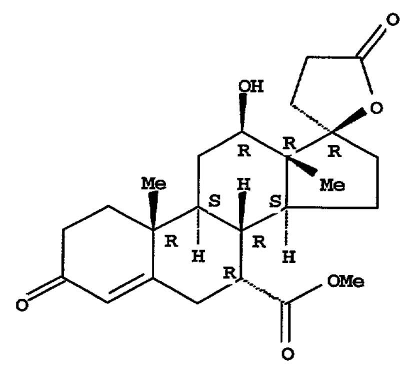
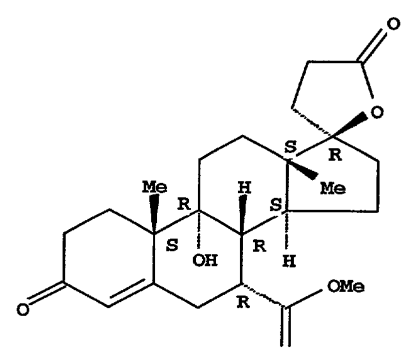
The term "substantially hybridizing" means that two nucleic acid molecules can form an anti-parallel, double-stranded nucleic acid structure under conditions (e.g., salt and temperature) that permit hybridization of sequences that exhibit 90% sequence identity or greater with each other and exhibit this identity for at least about a contiguous 50 nucleotides of the nucleic acid molecules.

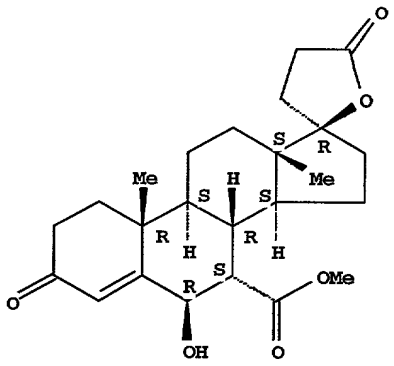
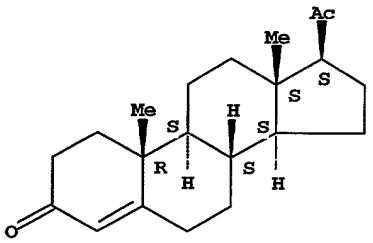
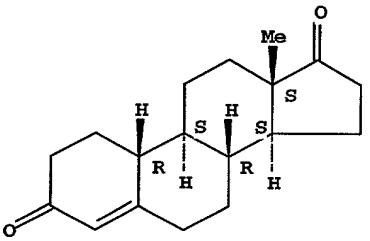
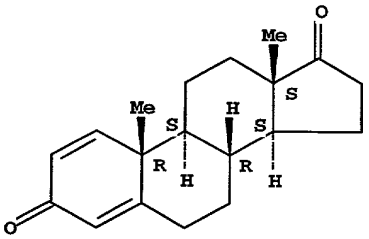
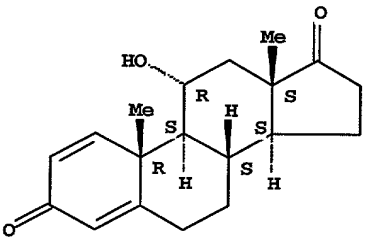
The term "substantially-purified" means that one or more molecules that are or may be present in a naturally-occurring preparation containing the target molecule will have been removed or reduced in concentration.

The following is a list of steroids, corresponding terms, and their structures, as used interchangeably herein:

#	Name	CA Index Name:	Other Names	Formula	Structure
1	Eplerenone	Pregn-4-ene-7,21-dicarboxylic acid, 9,11-epoxy-17-hydroxy-3-oxo-, γ-lactone, methyl ester, (7α,11α,17α)- (9CI)	Spiro[9,11-epoxy-9H-cyclopenta[a]phenanthrene-17(2H),2'(3'H)-furan], pregn-4-ene-7,21-dicarboxylic acid deriv.; CGP 30083; Eplerenone; SC 66110	C24 H30 O6	
2	Aldadiene; Canrenone	Pregna-4,6-diene-21-carboxylic acid, 17-hydroxy-3-oxo-, γ-lactone, (17α)- (9CI)	17α-Pregna-4,6-diene-21-carboxylic acid, 17-hydroxy-3-oxo-, γ-lactone (6CI, 7CI, 8CI); Spiro[17H-cyclopenta[a]phenanthrene-17,2'(5'H)-furan], pregna-4,6-diene-21-carboxylic acid deriv.; 11614 R.P.; 17β-Hydroxy-3-oxopregna-4,6-diene-21-carboxylic acid; 20-Spiroxa-4,6-diene-3,21-dione; Aldadiene;	C22 H28 O3	

			Canrenone; Phanurane; SC 9376; Spirolactone SC 14266		
3	11α-Hydroxycanrenone	Pregna-4,6-diene-21-carboxylic acid, 11,17-dihydroxy-3-oxo-, γ-lactone, (11α,17α)-(9CI)	11α-Hydroxycanrenone	C22 H28 O4	
5	Aldona ethyl enol ether	Pregna-4,6-diene-21-carboxylic acid, 3-ethoxy-17-hydroxy-, γ-lactone (9CI)	Spiro[17H-cyclopenta[a]phenanthrene-17,2'(5'H)-furan], pregna-4,6-diene-21-carboxylic acid deriv.; Aldona ethyl enol ether	C24 H34 O3	
6	Androstenedione	Androst-4-ene-3,17-dione (8CI, 9CI)	Δ4-Androstene-3,17-dione; 17-Ketotestosterone; 3,17-Dioxoandrost-4-ene; Androstenedione; Fecundin; SKF 2170	C19 H26 O2	
7	11α-Hydroxyandrostenedione	Androst-4-ene-3,17-dione, 11-hydroxy-, (11α)-(9CI)	Androst-4-ene-3,17-dione, 11α-hydroxy- (8CI); 11α-Hydroxyandrostendione; 11α-Hydroxyandrostenedione	C19 H26 O3	

8	Mexrenone	Pregn-4-ene-7,21-dicarboxylic acid, 17-hydroxy-3-oxo-, γ-lactone, methyl ester, (7α,17α)-(9CI)	Spiro[17H-cyclopenta[a]phenanthrene-17,2'(5'H)-furan], pregn-4-ene-7,21-dicarboxylic acid deriv.; Mexrenone; SC 25152; ZK 32055	$C_{24}H_{32}O_{5}$	
9	11β-Hydroxymexrenone	Pregn-4-ene-7,21-dicarboxylic acid, 11,17-dihydroxy-3-oxo-, γ-lactone, methyl ester, (7α,11β,17α)- (9CI)	11β-Hydroxymexrenone	$C_{24}H_{32}O_{6}$	
10	12β-Hydroxymexrenone	Pregn-4-ene-7,21-dicarboxylic acid, 12,17-dihydroxy-3-oxo-, γ-lactone, methyl ester, (7α,12β,17α)- (9CI)	12β-Hydroxymexrenone	$C_{24}H_{32}O_{6}$	
11	9α-Hydroxymexrenone	Pregn-4-ene-7,21-dicarboxylic acid, 9,17-dihydroxy-3-oxo-, 21,17-lactone, 7-methyl ester, (7α,17α)-(9CI)	9α-Hydroxymexrenone	$C_{24}H_{32}O_{6}$	

12	6β-Hydroxymexrenone	Pregn-4-ene-7,21-dicarboxylic acid, 6,17-dihydroxy-3-oxo-, γ-lactone, methyl ester, (6β,7α,17α)- (9CI)	Spiro[17H-cyclopenta[a]phenanthrene-17,2'(3'H)-furan], pregn-4-ene-7,21-dicarboxylic acid deriv.; 6β-Hydroxymexrenone	C24 H32 O6	
13	Progesterone	Pregn-4-ene-3,20-dione (9CI)	Progesterone (8CI); Δ4-Pregnene-3,20-dione; and >70 other names	C21 H30 O2	
14	Estr-4-ene-3,17-dione	Estr-4-ene-3,17-dione (6CI, 8CI, 9CI)	(+)-19-Norandrost-4-ene-3,17-dione; Δ4-Estrene-3,17-dione; 19-Norandrost-4-ene-3,17-dione	C18 H24 O2	
15	delta 1,4-androstadiene-3,17-dione (ADD)	Androsta-1,4-diene-3,17-dione (7CI, 8CI, 9CI)	Δ1,4-Androstadiene-3,17-dione; 1-Dehydroandrostenedione; Androstadienedione; Androstane-1,4-diene-3,17-dione	C19 H24 O2	
16	11α-Hydroxyandrosta-1,4-diene-3,17-dione (11 alpha hydroxy ADD)	Androsta-1,4-diene-3,17-dione, 11-hydroxy-, (11α)- (9CI)	Androsta-1,4-diene-3,17-dione, 11α-hydroxy- (6CI, 7CI, 8CI); 11α-Hydroxyandrosta-1,4-diene-3,17-dione; Kurchinin	C19 H24 O3	
17	aldona				Compound 5 (aldona ethyl enol ether) with O= in place of EtO- at position 3

18	mexrenone 6,7-bislactone	Compound 12 with cyclic bis-lactone ring (-O-C=O-) formed between carbons at positions 6 and 7 (See US 5,981,744 for discussion of similar lactone rings)
19	11 alpha hydroxy mexrenone 6,7-bislactone	11 alpha hydroxy version of Compound 18
20	mexrenone 7,9-bislactone	Compound 11 with cyclic bis-lactone ring (-O-C=O-) formed between carbons at positions 7 and 9 (See US 5,981,744 for discussion of similar lactone rings)
21	11 alpha hydroxy mexrenone 7,9-bislactone	11 alpha hydroxy version of Compound 20

Figure 1 - Nucleotide and protein sequence of *Aspergillus ochraceus* 11 alpha hydroxylase

The nucleotide and protein sequences of *Aspergillus ochraceus* 11 alpha hydroxylase (SEQ ID NO: 1, SEQ ID NO: 2, respectively) are displayed.

Figure 2 - Nucleotide and protein sequence of human oxidoreductase

The nucleotide and protein sequences of human oxidoreductase (SEQ ID NO: 3, SEQ ID NO: 4, respectively) are displayed. The predicted amino acid sequence of human oxidoreductase independently cloned from a cDNA library prepared by RT-PCR using the RNA from a human HepG2 cells as a template, as disclosed in this specification, matches that previous reported by three different laboratories. The GenBank accession numbers for these loci include A60557 (NADPH--ferrihemoprotein reductase (EC 1.6.2.4) - human); AAG09798 (NADPH-cytochrome P450 reductase [Homo sapiens]), and P16435 (NADPH-CYTOCHROME P450 REDUCTASE (CPR) (P450R)).

The amino acid sequence of AAB21814 (cytochrome P450 reductase {EC 1.6.2.4} [human, placenta, Peptide Partial, 676 aa]), differs from human oxidoreductase A60557 and P16435 at 4 residues: A→ V at 500, F→L at 518, V→W at 537, and A→H at 538. The initial methionine is also missing from AAB21814. The cognate nucleic acid for AA21814 (S90469 [cytochrome P450 reductase [human, placenta, mRNA Partial, 2403 nt]) lacks the ATG codon for the initial

methionine and includes a C→T change at 1496, a C→A, change at 1551, and a frameshift due to a missing G at 1605 which is resolved by the addition of a T at 1616.

References for these loci are as follows: A60557 [Yamano,S., Aoyama,T., McBride,O.W., Hardwick,J.P., Gelboin,H.V. and Gonzalez,F.J. Human NADPH-P450 oxidoreductase: complementary DNA cloning, sequence and vaccinia virus-mediated expression and localization of the CYPOR gene to chromosome 7 Mol. Pharmacol. 36 (1), 83-88 (1989)]; AAG09798 [Czerwinski,M., Sahni,M., Madan,A. and Parkinson,A. Polymorphism of human CYPOR: Expression of new allele. Unpublished, Direct Submission], and P16435 [Haniu,M., McManus,M.E., Birkett,D.J., Lee,T.D. and Shively,J.E. Structural and functional analysis of NADPH-cytochrome P-450 reductase from human liver: complete sequence of human enzyme and NADPH-binding sites. Biochemistry 28 (21), 8639-8645 (1989)]; AAB21814 [Shephard,E.A., Palmer,C.N., Segall,H.J. and Phillips,I.R. Quantification of cytochrome P450 reductase gene expression in human tissues. Arch. Biochem. Biophys. 294 (1), 168-172 (1992)]; S90469 [Shephard,E.A., Palmer,C.N., Segall,H.J. and Phillips,I.R. Quantification of cytochrome P450 reductase gene expression in human tissues. Arch. Biochem. Biophys. 294 (1), 168-172 (1992)].

Figure 3 - Nucleotide and protein sequence of *Aspergillus ochraceus* oxidoreductase

The nucleotide and protein sequences of *Aspergillus ochraceus* 11 oxidoreductase (SEQ ID NO: 5, SEQ ID NO: 6, respectively) are displayed.

Figure 4 - Amino acid homology alignment of *A. ochraceus* 11 alpha hydroxylase with the top 10 BLAST hits from GenBank

Aspergillus ochraceus steroid 11 alpha hydroxylase (SEQ ID NO: 02), cloned into plasmid pMON45624 (SEQ ID NO: 01), was aligned with related enzymes found in GenBank using the BLASTP program that implements a heuristic matching algorithm (Altschul et al., *J Mol Biol* Oct 5;215(3):403-10, 1990). The GenBank accession numbers (its probable function, [*genus* and *species*]) for the top 10 matches are as follows: CAA75565 (cytochrome P450 monooxygenase [*Gibberella fujikuroi*]; CAB91316 (probable cytochrome P450 monooxygenase (*lov*A) [*Neurospora crassa*]); CAB56503 (cytochrome P450 [*Catharanthus roseus*]); AAB94588 (CYP71D10p [*Glycine max*]); CAA75566 (cytochrome P450 monooxygenase [*Gibberella fujikuroi*]); AAD34552 (cytochrome P450

monooxygenase [*Aspergillus terreus*]); CAA75567 (cytochrome P450 monooxygenase [*Gibberella fujikuroi*]); CAA76703 (cytochrome P450 [*Gibberella fujikuroi*]); CAA57874 (unnamed protein product [*Fusarium oxysporum*]); CAA91268 (similar to cytochrome P450~cDNA EST yk423b11.3 comes from this gene [*Caenorhabditis elegans*]).

References for these loci are as follows: CAA75565 [Tudzynski,B. and Holter,K., Gibberellin biosynthetic pathway in *Gibberella fujikuroi*: evidence for a gene cluster. *Fungal Genet. Biol.* **25** (3), 157-170 (1998)]; CAB91316 [Schulte,U., Aign,V., Hoheisel,J., Brandt,P., Fartmann,B., Holland,R., Nyakatura,G., Mewes,H.W. and Mannhaupt,G., Unpublished]; CAB56503 [Schroeder,G., Unterbusch,E., Kaltenbach,M., Schmidt,J., Strack,D. and Schroeder,J. Light-induced cytochrome P450-dependent enzyme in indole alkaloid biosynthesis: tabersonine 16-hydroxylase *FEBS Lett.* **458**, 97-102 (1999)]; AAB94588 [Siminszky,B., Corbin,F.T., Ward,E.R., Fleischmann,T.J. and Dewey,R.E. Expression of a soybean cytochrome P450 monooxygenase cDNA in yeast and tobacco enhances the metabolism of phenylurea herbicides. *Proc. Natl. Acad. Sci. U.S.A.* **96** (4), 1750-1755 (1999)]; CAA75566 [Tudzynski,B. and Holter,K. Gibberellin biosynthetic pathway in *Gibberella fujikuroi*: evidence for a gene cluster. *Fungal Genet. Biol.* **25** (3), 157-170 (1998)]; AAD34552 [Kennedy,J., Auclair,K., Kendrew,S.G., Park,C., Vederas,J.C. and Hutchinson,C.R. Accessory Proteins Modulate Polyketide Synthase Activity During Lovastatin Biosynthesis. *Science* (1999) In press]; CAA75567 [Tudzynski,B. and Holter,K. Gibberellin biosynthetic pathway in *Gibberella fujikuroi*: evidence for a gene cluster. *Fungal Genet. Biol.* **25** (3), 157-170 (1998)]; CAA76703 [Tudzynski,B. and Hoelter,K. Characterization of P450 monooxygenase genes from *Gibberella fujikuroi*. Unpublished]; CAA57874 [Mouyna,I. and Brygoo,Y. Disruption of a *Fusarium oxysporum f.sp. elaeidis* cytochrome P450 gene by a repetitive sequence. Unpublished]; and CAA91268 [No Authors. Genome sequence of the nematode *C. elegans*: a platform for investigating biology. The *C. elegans* Sequencing Consortium. Science **282** (5396), 2012-2018 (1998) [Published errata appear in *Science* 1999 Jan 1;**283**(5398):35 and 1999 Mar 26;**283**(5410):2103 and 1999 Sep 3;**285**(5433):1493]]].

Figure 5 – Phylogenetic tree showing the relatedness of *Aspergillus ochraceus* 11 alpha hydroxylase to the top 10 BLAST hits from GenBank

A phylogenetic tree displaying the genetic relatedness of *Aspergillus ochraceus* steroid 11 alpha hydroxylase, cloned into plasmid pMON45624, was

aligned with related enzymes found in GenBank. BLAST was used to find the related enzymes within GenBank, and ClustalW was used generate the multiple sequence alignment and phylogenetic tree depicted in this figure. Descriptions of the GenBank accession numbers used as labels in the figure are the same as that described above for the legend to Figure 4.

Figure 6 – Percent homology between *Aspergillus ochraceus* 11 alpha hydroxylase and the top 10 BLAST hits from GenBank

The percent homology between *Aspergillus ochraceus* steroid 11 alpha hydroxylase and the top 10 enzymes found in GenBank using BLAST was calculated using CLUSTAL (Thompson et al., *Comput. Appl. Biosci.* **10**:19-29, 1994).

Figure 7 - Amino acid homology alignment of *Aspergillus ochraceus* and human oxidoreductase to NADPH cytochrome P450 reductases from *A. niger*, mouse, and *S. cerevisiae*

The amino acid sequences of *Aspergillus ochraceus* steroid oxidoreductase (SEQ ID NO: 06) cloned into plasmid pMON45632 (SEQ ID NO: 05), and human oxidoreductase (SEQ ID NO: 03), cloned into plasmid pMON45605 (SEQ ID NO: 04) were aligned with related enzymes from *A. niger*, mouse, and *S. cervisiase*, as described above. The GenBank accession numbers (probable function, [genus and species]) are as follows: BAA02936 (NADPH-cytochrome P450 reductase precursor [*Saccharomyces cerevisiae*]); CAA81550 NADPH cytochrome P450 oxidoreductase [*Aspergillus niger*]; P16435 (NADPH-CYTOCHROME P450 REDUCTASE (CPR) (P450R) [human]); BAA04496 (NADPH-cytochrome P450 oxidoreductase [*Mus musculus*]).

References for these loci are as follows: BAA02936 [Yabusaki,Y., Murakami,H. and Ohkawa,H. Primary structure of *Saccharomyces cerevisiae* NADPH-cytochrome P450 reductase deduced from nucleotide sequence of its cloned gene. *J. Biochem.* 103 (6), 1004-1010 (1988)]; CAA81550 [van den Brink,J., van Zeijl,C., van den Hondel,C. and van Gorcom,R. Cloning and characterization of the NADPH cytochrome P450 oxidoreductase (cprA) gene of *Aspergillus niger*. Unpublished]; P16435 [Haniu,M., McManus,M.E., Birkett,D.J., Lee,T.D. and Shively,J.E. Structural and functional analysis of NADPH-cytochrome P-450 reductase from human liver: complete sequence of human enzyme and NADPH-binding sites *Biochemistry* 28 (21), 8639-8645 (1989)]; BAA04496 [Ohgiya,S., Shinriki,N., Kamataki,T. and Ishizaki,K. Mouse NADPH-cytochrome P-450

oxidoreductase: molecular cloning and functional expression in yeast. *Biochim. Biophys. Acta* 1186 (1-2), 137-141 (1994)].

Figure 8 – Amino acid homology alignment of A. ochraceus oxidoreductase to NADPH cytochrome P450 reductases from *A. niger*, mouse, and *S. cerevisiae*

The amino acid sequence of *Aspergillus ochraceus* steroid oxidoreductase (SEQ ID NO: 06) cloned into plasmid pMON45632 (SEQ ID NO: 05), was aligned with related fungal enzymes from *A. niger* and *S. cervisiase*, as described above. Descriptions of the GenBank accession numbers used as labels in the figure are the same as that described above for the legend to Figure 7, above.

Figure 9 - Phylogenetic tree showing the relatedness of *Aspergillus ochraceus* and human oxidoreductase to reductases from *A. niger*, yeast, and mouse.

A phylogenetic tree displaying the genetic relatedness of Aspergillus ochraceus oxidoreductase (SEQ ID NO: 06), cloned into plasmid pMON45632 (SEQ ID NO: 05), was aligned with related enzymes. BLAST was used to find the related enzymes within GenBank, and ClustalW was used generate the multiple sequence alignment and phylogenetic tree depicted in this figure. Descriptions of the GenBank accession numbers used as labels in the figure are the same as that described above for the legend to Figure 7, above.

Figure 10 - Percent identity between *Aspergillus ochraceus* oxidoreductase and reductases from *A. niger*, yeast, and mouse.

The percent identity between *Aspergillus ochraceus* oxidoreductase and the oxidoreductases from *A. niger*, yeast, and mouse was calculated using Clustal W and Boxshade.

Figure 11 - Alignment of human oxidoreductase with top 4 hits from SwissProt

The amino acid sequences of human steroid oxidoreductase (SEQ ID NO: 04), cloned into plasmid pMON45605 (SEQ ID NO: 03), which corresponds to the amino acid sequence of the corrected sequence reported for P16435 below, was aligned with the top 4 hits from the SWISSPROT protein sequence database, as described above. The SWISSPROT accession numbers {locus} [common name] and

species]) probable function) are as follows: P16435 {NCPR_HUMAN} [human] NADPH-CYTOCHROME P450 REDUCTASE; P00389 {NCPR_RABIT} [rabbit] NADPH-CYTOCHROME P450 REDUCTASE; P00388 {NCPR_RAT} [rat] NADPH-CYTOCHROME P450 REDUCTASE; P37040 {NCPR_MOUSE} [mouse] NADPH-CYTOCHROME P450 REDUCTASE; P04175 {NCPR_PIG} [pig] (NADPH-CYTOCHROME P450 REDUCTASE.

References for these loci are as follows: P16435 [Haniu,M., McManus,M.E., Birkett,D.J., Lee,T.D. and Shively,J.E. Structural and functional analysis of NADPH-cytochrome P-450 reductase from human liver: complete sequence of human enzyme and NADPH-binding sites. *Biochemistry* 28 (21), 8639-8645 (1989)]; P00389 [Katagiri,M., Murakami,H., Yabusaki,Y., Sugiyama,T., Okamoto,M., Yamano,T. and Ohkawa,H. Molecular cloning and sequence analysis of full-length cDNA for rabbit liver NADPH-cytochrome P-450 reductase mRNA. *J. Biochem.* 100 (4), 945-954 (1986)]; P00388 [Porter,T.D. and Kasper,C.B. Coding nucleotide sequence of rat NADPH-cytochrome P-450 oxidoreductase cDNA and identification of flavin-binding domains. *Proc. Natl. Acad. Sci. U.S.A.* 82 (4), 973-977 (1985)]; P37040 [Ohgiya,S., Shinriki,N., Kamataki,T. and Ishizaki,K. Mouse NADPH-cytochrome P-450 oxidoreductase: molecular cloning and functional expression in yeast. *Biochim. Biophys. Acta* 1186 (1-2), 137-141 (1994)]; P04175 [Haniu,M., Iyanagi,T., Miller,P., Lee,T.D. and Shively,J.E. Complete amino acid sequence of NADPH-cytochrome P-450 reductase from porcine hepatic microsomes. *Biochemistry* 25 (24), 7906-7911 (1986)].

Figure 12 – Phylogenetic tree showing the relatedness of human oxidoreductases with top 4 hits from SwissProt

A phylogenetic tree displaying the genetic relatedness of human oxidoreductase (SEQ ID NO: 04), cloned into plasmid pMON45604 (SEQ ID NO: 03), was aligned with related enzymes found in SWISSPROT. BLAST was used to find the related enzymes within SWISSPROT, and ClustalW was used generate the multiple sequence alignment and phylogenetic tree depicted in this figure. Descriptions of the SWISPROT accession numbers used as labels in the figure are the same as that described above for the legend to Figure 11, above.

Figure 13 – Percent identity between human oxidoreductase and top 4 hits from SwissProt

The percent identity between human oxidoreductase and the top 4 hits found in SWISSPROT was calculated using Clustal W and Boxshade.

Figure 14: Expression of *Aspergillus ochraceus* 11 alpha hydroxylase in transfected Sf9 insect cells

Baculovirus-infected insect cells expressing *Aspergillus ochraceus* 11 alpha hydroxylase were harvested at 25 and 48 hours post infection and microsomal membrane fractions were prepared and separated by SDS-polyacrylamide gel electrophoresis. The proteins in the gel were electrophoretically transferred to 0.2 um nitrocellulose membrane (Schleicher & Schuell Grimsehlstrasse 23 37574 Einbeck Germany) and probed with antibodies GN-1187 and GN-1188 prepared from peptide 11aOH peptide 2 CRQILTPYIHKRKSLKGTTD (SEQ ID NO: 24).

Figure 15: Expression of *Aspergillus ochraceus* P450 oxidoreductase in transfected Sf9 insect cells

Baculovirus-infected insect cells expressing *Aspergillus ochraceus* 11 oxidoreductase were harvested at 25 and 48 hours post infection and microsomal membrane fractions were prepared and separated by SDS-polyacrylamide gel electrophoresis. The proteins in the gel were electrophoretically transferred to 0.2 um nitrocellulose membrane (Schleicher & Schuell Grimsehlstrasse 23 37574 Einbeck Germany) and probed with antibodies GN-2023 and GN-12024 prepared from oxr peptide 1 CTYWAVAKDPYASAGPAMNG (SEQ ID NO: 26).

Figure 16 – Conversion of androstenedione to 11 alpha hydroxy androstenedione monitored by HPLC

Microsomal and mitochondrial subcellular fractions were prepared from insect cells co-infected with recombinant baculoviruses expressing recombinant *Aspergillus ochraceus* 11 alpha hydroxylase and human oxidoreductase cloned from HepG2 cell RNA. The subcellular fractions were incubated with 250 μM androstenedione (AD) in the presence of an NADPH-generating system for 120 minutes, and the resulting products were separated by HPLC and monitored by ultraviolet detection at 247 nm. Hydroxlase activity was found in the microsomal fraction, as expected, but also appeared in the mitochondrial fraction. These results suggest that the 11 alpha hydroxylase may have a tendency to stick to membranes in disrupted cells, or that the separation of the subcellular fractions in this experiment was insufficient. Panel A illustrates a reaction carried out using enzyme prepared from a mitochondrial fraction. The peak in panel A that elutes after AD appears to be testosterone. When a microsomal fraction was used, almost as much AD was converted to 11 alpha hydroxy AD, but relatively more

testosterone was also produced. Panel B illustrates the same reaction carried out for 120 minutes without a source of enzyme. Panel C illustrates an HPLC tracing with 11α-hydroxyandrostenedione standard added to incubation buffer.

Detailed Description of the Invention

The present invention encompasses enzymes that facilitate the biosynthesis of steroid molecules, particularly enzymes possessing cytochrome P450 or oxidoreductase activities. The present invention is directed, in part, to the isolation of a nucleic acid encoding *Aspergillus ochraceus* 11 alpha hydroxylase, which exhibits sequence homology to the highly conserved residues that correspond to cytochrome P450 enzymes. It also directed to the isolation of nucleic acids encoding human and *Aspergillus ochraceus* oxidoreductase. Biological activities of the cloned hydroxylases and oxidoreductases of the present invention can be determined by a variety of assays, including incubation of steroid substrates in the presence of microsomes prepared from recombinant baculovirus-infected insect cells and monitoring the conversion to their 11 alpha hydroxy-counterparts by high pressure liquid chromatography (HPLC). The present invention, comprising novel 11 alpha hydroxylase and oxidoreductase nucleic acids, proteins, peptides, homologues, and fragments of either, provides new and advantageous methods to convert steroid intermediates to their 11 alpha hydroxy counterparts.

The present invention also includes the DNA sequences which code for the 11 alpha hydroxylases and oxidoreductases, DNA sequences which are substantially similar and perform substantially the same function, and DNA sequences which differ from the DNAs encoding the hydroxylases and oxidoreductases of the invention only due to the degeneracy of the genetic code. Also included in the present invention are the oligonucleotide intermediates used to construct mutated versions of these DNAs and the polypeptides encoded by these oligonucleotides and mutant DNAs.

The present invention also includes antibodies which bind specifically to *A. ochraceus* 11 alpha hydroxylase or *A. ochraceus* oxidoreductase, including anti-peptide antibodies, methods of using these anti-peptide antibodies to purify these and other related polypeptides, methods of using the purified polypeptides to generate polyclonal or monoclonal antibodies to the full-length polypeptides, and methods of using antibodies to the full-length polypeptides to assess the presence of the polypeptides in recombinant and non-recombinant host cells. The antibodies

can be used to identify related polypeptides in any of a variety of host organisms that possess the biological activities associated with these polypeptides.

Among the preferred organisms that can be used in this hydroxylation step are *Aspergillus ochraceus* NRRL 405, *Aspergillus ochraceus* ATCC 18500, *Aspergillus niger* ATCC 16888 and ATCC 26693, *Aspergillus nidulans* ATCC 11267, *Rhizopus oryzae* ATCC 11145, *Rhizopus stolonifer* ATCC 6227b, *Streptomyces fradiae* ATCC 10745, *Bacillus megaterium* ATCC 14945, *Pseudomonas cruciviae* ATCC 13262, and *Trichothecium roseum* ATCC 12543. Other preferred organisms include *Fusarium oxysporum f. sp. cepae* ATCC 11171 and *Rhizopus arrhizus* ATCC 11145.

Other organisms that have exhibited activity for this reaction include *Absidia coerula* ATCC 6647, *Absidia glauca* ATCC 22752, *Actinomucor elegans* ATCC 6476, *Aspergillus flavipes* ATCC 1030, *Aspergillus fumigatus* ATCC 26934, *Beauveria bassiana* ATCC 7159 and ATCC 13144, *Botryosphaeria obtusa* IMI 038560, *Calonectria decora* ATCC 14767, *Chaetomium cochliodes* ATCC 10195, *Corynespora cassiicola* ATCC 16718, *Cunninghamella blakesleeana* ATCC 8688a, *Cunninghamella echinulata* ATCC 3655, *Cunninghamella elegans* ATCC 9245, *Curvularia clavata* ATCC 22921, *Curvularia lunata* ACTT 12071, *Cylindrocarpon radicicola* ATCC 1011, *Epicoccum humicola* ATCC 12722, *Gongronella butleri* ATCC 22822, *Hypomyces chrysospermus*, *Mortierella isabellina* ATCC 42613, *Mucor mucedo* ATCC 4605, *Mucor griseocyanus* ATCC 1207A, *Myrothecium verrucaria* ATCC 9095, *Nocardia corallina*, *Paecilomyces carneus* ATCC 46579, *Penicillum patulum* ATCC 24550, *Pithomyces atroolivaceus* IFO 6651, *Pithomyces cynodontis* ATCC 26150, *Pycnosporium* sp. ATCC 12231, *Saccharopolyspora erythrae* ATCC 11635, *Sepedonium chrysospermum* ATCC 13378, *Stachylidium bicolor* ATCC 12672, *Streptomyces hyqroscopicus* ATCC 27438, *Streptomyces purpurascens* ATCC 25489, *Syncephalastrum racemosum* ATCC 18192, *Thamnostylum piriforme* ATCC 8992, *Thielavia terricola* ATCC 13807, and *Verticillium theobromae* ATCC 12474.

Additional organisms that may be expected to show activity for the 11α hydroxylation include *Cephalosporium aphidicola* (*Phytochemistry* (1996), **42**(2), 411-415), *Cochliobolus lunatas* (*J. Biotechnol.* (1995), 42(2), 145-150), *Tieghemella orchidis* (*Khim.-Farm.Zh.* (1986), 20(7), 871- 876), *Tieghemella hyalospora* (*Khim.-Farm.Zh.* (1986), 20(7), 871-876), *Monosporium olivaceum* (*Acta Microbiol. Pol.*, Ser. B. (1973), 5(2), 103-110), *Aspergillus ustus* (*Acta Microbiol. Pol.*, Ser. B. (1973), 5(2), 103-110), *Fusarium graminearum* (*Acta Microbiol. Pol.*, Ser. B. (1973), 5(2),

103-110), *Verticillium glaucum* (*Acta Microbiol. Pol.*, Ser. B. (1973), 5(2), 103-110), and *Rhizopus nigricans* (*J. Steroid Biochem.* (1987), 28(2), 197-201).

Figure 1 sets forth the nucleotide and protein sequence of *Aspergillus ochraceus* 11 alpha hydroxylase (SEQ ID NO: 1, SEQ ID NO: 2, respectively). Figure 2 sets forth the nucleotide and protein sequence of human oxidoreductase (SEQ ID NO: 3, SEQ ID NO: 4, respectively). Figure 3 sets forth the nucleotide and protein sequence of *Aspergillus ochraceus* oxidoreductase (SEQ ID NO: 5, SEQ ID NO: 6, respectively).

Figure 4 sets forth an amino acid homology alignment of *A. ochraceus* 11 alpha hydroxylase cloned in pMON45624 and aligned with related enzymes found in GenBank using BLAST. Figure 5 is a phylogenetic tree showing the this relationship graphically. Figure 6 shows the percent homology between *Aspergillus ochraceus* steroid 11 alpha hydroxylase and the top 10 enzymes found in GenBank using BLAST, calculated using Clustal W and Boxshade.

Figure 7 sets forth the amino acid homology of *Aspergillus ochraceus* and human oxidoreductase to NADPH cytochrome P450 reductases from *A. niger*, mouse, and *S. cerevisiae* (yeast). Figure 8 sets forth the amino acid alignment for *A. ochraceus, A. niger,* and *S. cerevisiae* oxidoreductases. Figure 9 is a phylogenetic tree showing the relatedness of *Aspergillus ochraceus* and human oxidoreductase to reductases from *A. niger*, yeast, and mouse. Figure 10 shows the percent homology between *Aspergillus ochraceus* steroid 11 alpha hydroxylase and the oxidoreductases from *A. niger*, yeast, and mouse, calculated using Clustal W and Boxshade.

Figure 11 - Alignment of human oxidoreductase with top 4 hits from SwissProt. Figure 12 sets forth a phylogenetic tree displaying the genetic relatedness of human oxidoreductase, to these hits. Figure 13 shows the percent identity between human oxidoreductase and top 4 hits from SwissProt.

Figure 14 sets forth an immunoblot illustrating expression of *Aspergillus ochraceus* P450 11 alpha hydroxylase in baculovirus-infected insect cells harvested at 25 and 48 hours post infection. The nitrocellulose membrane was probed with a 1:1 mixture of antibodies prepared from two rabbits immunized with a conjugated synthetic peptide, 11aOH peptide 2 (SEQ ID NO 24).

Figure 15 sets forth an immunoblot illustrating expression of *Aspergillus ochraceus* P450 oxidoreductase in baculovirus-infected insect cells harvested at 25

and 48 hours post infection. The nitrocellulose membrane was probed with a 1:1 mixture of antibodies prepared two rabbits immunized with a conjugated synthetic peptide, oxr peptide 1 (SEQ ID NO 26).

Figure 16 sets forth an HPLC tracing illustrating the conversion of androstenedione (AD) to its 11 alpha hydroxy counterpart after incubating AD with subcellular fractions prepared from baculovirus-infected insect cells expressing *Aspergillus ochraceus* 11 alpha hydroxylase and human oxidoreductase.

Cloning techniques

Genetic engineering techniques now standard in the art (U.S. Patent 4,935,233 and Sambrook et al., "Molecular Cloning A Laboratory Manual", Cold Spring Harbor Laboratory, 1989) may be used in the construction of the DNA sequences of the present invention. One such method is cassette mutagenesis (Wells et al., *Gene* **34**:315-323, 1985) in which a portion of the coding sequence in a plasmid is replaced with synthetic oligonucleotides that encode the desired amino acid substitutions in a portion of the gene between two restriction sites.

Pairs of complementary synthetic oligonucleotides encoding the desired gene can be made and annealed to each other. The DNA sequence of the oligonucleotide would encode sequence for amino acids of desired gene with the exception of those substituted and/or deleted from the sequence.

Plasmid DNA can be treated with the chosen restriction endonucleases then ligated to the annealed oligonucleotides. The ligated mixtures can be used to transform competent *E. coli* cells which will confer resistance to an appropriate antibiotic. Single colonies can be picked and the plasmid DNA examined by restriction analysis or by DNA sequencing to identify plasmids with the desired genes.

Cloning of DNA sequences encoding novel proteins and fusion proteins may be accomplished by the use of intermediate vectors. Linkers and adapters can be used to join DNA sequences, and to replace lost sequences, where a restriction site is internal to the region of interest. DNA encoding a single polypeptide or a fusion protein (comprising a first polypeptide, a peptide linker, and a second polypeptide) is inserted into a suitable expression vector which is then transformed or transfected into appropriate bacterial, fungal, insect, or mammalian host cells. The transformed organism or host cell line is grown and the recombinant protein

isolated by standard techniques. Recombinant fusion proteins have all or a portion of a first protein joined by a linker region to a all or a portion of second protein.

Hybridization

Nucleic acid molecules and fragment nucleic acid molecules encoding 11 alpha hydroxylases or oxidoreductases can specifically hybridize with other nucleic acid molecules. Two nucleic acid molecules are said to be capable of specifically hybridizing to one another if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure. A nucleic acid molecule is said to be the "complement" of another nucleic acid molecule, if they exhibit complete complementarity. Molecules exhibit "complete complementarity" when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are "minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low-stringency" conditions. Similarly, the molecules are "complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high-stringency" conditions. Conventional stringency conditions are described by Sambrook, *et al.*, *Molecular Cloning,* A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, New York (1989), and by Haymes, *et al. Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, DC, 1985). Departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure.

Appropriate stringency conditions which promote DNA hybridization are well known to those skilled in the art, or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y., 6.3.1-6.3.6, (1989). Basic conditions would include, for example, 6X sodium saline citrate (SSC) at about 45°C, followed by a wash of 2X SSC at 50°C. Stringency can be varied, for example, by altering the salt concentration in the wash step from about 2X SSC at 50°C (moderately low stringency) to about 0.2X SSC at 50°C (high stringency). Stringency can also be altered by changing the temperature in the wash step, from room temperature, about 22°C (low stringency conditions), to about 65°C (high stringency conditions). Both temperature and salt may be varied, or either the temperature or the salt concentration may be held constant while the other variable is changed.

Expression vectors

Another aspect of the present invention includes plasmid DNA vectors for use in the expression of these novel hydroxylases and oxidoreductases. These vectors contain the novel DNA sequences described above which code for the novel polypeptides of the invention. Appropriate vectors which can transform microorganisms or cell lines capable of expressing the hydroxylases and oxidoreductases include expression vectors comprising nucleotide sequences coding for the hydroxylases and oxidoreductases joined to transcriptional and translational regulatory sequences which are selected according to the host cells used.

Vectors incorporating modified sequences as described above are included in the present invention and are useful in the production of the hydroxylases and oxidoreductases. The vector employed in the method also contains selected regulatory sequences in operative association with the DNA coding sequences of the invention and which are capable of directing the replication and expression thereof in selected host cells.

Methods for producing the hydroxylases and oxidoreductases is another aspect of the present invention. The method of the present invention involves culturing suitable cells or cell lines, which has been transformed with a vector containing a DNA sequence encoding novel hydroxylases and oxidoreductases. Suitable cells or cell lines may be bacterial cells. For example, various strains of *E. coli* are well-known as host cells in the field of biotechnology. Examples of such strains include *E. coli* strains DH5 alpha, DH10B and MON105 (Obukowicz et al., *Applied Environmental Microbiology* **58**: 1511-1523, 1992). Also included in the present invention is the expression of the hydroxylases and oxidoreductases utilizing a chromosomal expression vector for *E. coli* based on the bacteriophage Mu (Weinberg et al., *Gene* **126**: 25-33, 1993). Various other strains of bacteria, including the Enteric bacteria (e.g., *Salmonella* sp.) and *B. subtilis,* may also be employed in this method.

When expressed in the *E. coli* cytoplasm, the gene encoding the proteins of the present invention may also be constructed such that at the 5' end of the gene codons are added to encode Met^{-2}-Ala^{-1}, Met^{-2}-Ser^{-1}, Met^{-2}-Cys^{-1}, or Met^{-1} at the N-terminus of the protein. The N termini of proteins made in the cytoplasm of *E. coli* are affected by post-translational processing by methionine aminopeptidase (Ben Bassat et al., *J. Bacteriol.* **169:**751-757, 1987), and possibly by other peptidases, so

that upon expression the methionine is cleaved off the N-terminus. The proteins of the present invention may include polypeptides having Met^{-1}, Ala^{-1}, Ser^{-1}, Cys^{-1}, Met^{-2}-Ala^{-1}, Met^{-2}-Ser^{-1}, or Met^{-2}-Cys^{-1} at the N-terminus. These mutant proteins may also be expressed in *E. coli* by fusing a secretion signal peptide to the N-terminus. This signal peptide is cleaved from the polypeptide as part of the secretion process.

Yeast

Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention. Under another embodiment, the protein or fragment thereof of the present invention is expressed in a yeast cell, preferably *Saccharomyces cerevisiae*. The proteins or fragments thereof of the present invention can be expressed in *S. cerevisiae* by fusing it to the N-terminus of the URA3, CYC1 or ARG3 genes (Guarente and Ptashne, *Proc. Natl. Acad. Sci. (U.S.A.) 78*:2199-2203 (1981); Rose *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.) 78*:2460-2464 (1981); and Crabeel *et al.*, *EMBO J. 2*:205-212 (1983)). Alternatively, proteins or fragments thereof of the present invention can be fused to either the PGK or TRP1 genes (Tuite *et al.*, *EMBO J. 1*:603-608 (1982); and Dobson *et al.*, *Nucleic Acids. Res. 11*:2287-2302 (1983)). More preferably, the protein or fragment thereof of the present invention is expressed as a mature protein (Hitzeman *et al.*, *Nature 293*:717-722 (1981); Valenzuela *et al.*, *Nature 298*:347-350 (1982); and Derynck *et al.*, *Nucleic Acids Res. 11*:1819-1837 (1983)).

Native and engineered yeast promoters suitable for use in the present invention have been reviewed by Romanos *et al.*, *Yeast 8*:423-488 (1992). Most preferably, the protein or fragment thereof of the present invention is secreted by the yeast cell (Blobel and Dobberstein, *J. Cell Biol. 67*:835-851 (1975); Kurjan and Herskowitz, *Cell 30*:933-943 (1982); Bostian *et al.*, *Cell 36*:741-751 (1984); Rothman and Orci, *Nature 355*:409-415 (1992); Julius *et al.*, *Cell 32*:839-852 (1983); and Julius *et al.*, *Cell 36*:309-318 (1984)).

Mammalian

General methods for expression of foreign genes in mammalian cells have been reviewed (Kaufman, R. J., 1987, "Genetic Engineering, Principles and Methods", Vol. 9, J. K. Setlow, editor, Plenum Press, New York; Colosimo et al., *Biotechniques* **29**: 314-331, 2000). Recombinant proteins are generally targeted to their natural locations within the host cell (e.g., cytoplasm, nucleus, or various

membrane compartments), or are secreted, if a signal peptide is present. An expression vector is constructed in which a strong promoter capable of functioning in mammalian cells drives transcription of a eukaryotic secretion signal peptide coding region, which is translationally joined to the coding region for the desired protein. For example, plasmids such as pcDNA I/Neo, pRc/RSV, and pRc/CMV (obtained from Invitrogen Corp., San Diego, California) can be used. The eukaryotic secretion signal peptide coding region can be from the gene itself or it can be from another secreted mammalian protein (Bayne, M. L. et al., *Proc. Natl. Acad. Sci. USA* **84**: 2638-2642, 1987). After construction of the vector containing the gene, the vector DNA is transfected into mammalian cells such as the COS7, HeLa, BHK, Chinese hamster ovary (CHO), or mouse L lines. The cells can be cultured, for example, in DMEM media (JRH Scientific). The polypeptide secreted into the media can be recovered by standard biochemical approaches following transient expression for 24 - 72 hours after transfection of the cells or after establishment of stable cell lines following selection for antibiotic resistance. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Gething and Sambrook, *Nature*, **293**:620-625, 1981, or alternatively, Kaufman et al, *Mol. Cell. Biol.*, **5(7)**:1750-1759, 1985) or Howley et al., and U.S. Pat. No. 4,419,446. Other suitable mammalian cell lines are the monkey COS-1 cell line and the CV-1 cell line.

Mammalian cells can also be used to express the nucleic acid molecules of the present invention. The nucleic acid molecules of the present invention can be cloned into a suitable retroviral vector (*see, e.g.,* Dunbar *et al.*, *Blood 85*:3048-3057 (1995); Baum *et al.*, *J. Hematother. 5*: 323-329 (1996); Bregni *et al.*, *Blood 80*:1418-1422 (1992); Boris-Lawrie and Temin, *Curr. Opin. Genet. Dev. 3*:102-109 (1993); Boris-Lawrie and Temin, *Annal. New York Acad. Sci. 716*:59-71 (1994); Miller, *Current Top. Microbiol. Immunol. 158*:1-24 (1992)), adenovirus vector (Berkner, *BioTechniques 6*:616-629 (1988); Berkner, *Current Top. Microbiol. Immunol. 158*:39-66 (1992); Brody and Crystal, *Annal. New York Acad. Sci. 716*:90-103 (1994); Baldwin *et al.*, *Gene Ther. 4*:1142-1149 (1997)), RSV, MuSV, SSV, MuLV (Baum *et al.*, *J. Hematother. 5*: 323-329 (1996)), AAV (Chen *et al., Gene Ther. 5*:50-58 (1998); Hallek *et al.*, *Cytokines Mol. Ther. 2*: 69-79 (1996)), AEV, AMV, or CMV (Griffiths *et al.*, *Biochem. J. 241*: 313-324 (1987)).

Transformation and transfection

In another aspect, the invention provides a transformed cell having a nucleic acid molecule which comprises an exogenous promoter region which functions in a cell to cause the production of an mRNA molecule which is linked to a structural nucleic acid molecule, wherein the structural nucleic acid molecule encodes an 11 alpha hydroxylase or oxidoreductase gene or fragment thereof. This nucleic acid molecule is linked to a 3' non-translated sequence that functions in a cell to cause termination of transcription and addition of polyadenylated ribonucleotides to a 3' end of the mRNA molecule.

Methods and compositions for transforming eukaryotic cells, bacteria and other microorganisms are known in the art (*see*, for example, Sambrook *et al., Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1989); Colosimo et al., *Biotechniques* **29**: 314-331, 2000).

Technology for introduction of DNA into cells is well known to those of skill in the art. Four general methods for delivering a gene into cells have been described: (1) chemical methods (Graham and van der Eb, *Virology 54*:536-539 (1973)); (2) physical methods such as microinjection (Capecchi, *Cell* 22:479-488 (1980)), electroporation (Wong and Neumann, *Biochem. Biophys. Res. Commun. 107*:584-587 (1982); Fromm *et al., Proc. Natl. Acad. Sci. (U.S.A.) 82*:5824-5828 (1985); U.S. Patent No. 5,384,253); and the gene gun (Johnston and Tang, *Methods Cell Biol. 43*:353-365 (1994); (3) viral vectors (Clapp, *Clin. Perinatol. 20*:155-168 (1993); Lu *et al., J. Exp. Med. 178*:2089-2096 (1993); Eglitis and Anderson, *Biotechniques, 6*:608-614 (1988)); and (4) receptor-mediated mechanisms (Curiel *et al., Hum. Gen. Ther. 3*:147-154 (1992), Wagner *et al., Proc. Natl. Acad. Sci. (U.S.A.) 89*:6099-6103 (1992)). Other methods well known in the art can also be used.

Transformation can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments (see for example Potrykus *et al., Mol. Gen. Genet. 205*:193-200 (1986); Lorz *et al., Mol. Gen. Genet. 199*:178 (1985); Fromm *et al., Nature 319*:791 (1986); Uchimiya *et al., Mol. Gen. Genet. 204*:204 (1986); Marcotte *et al., Nature 335*:454-457 (1988)).

Assays for gene expression based on the transient expression of cloned nucleic acid constructs have been developed by introducing the nucleic acid molecules into cells by polyethylene glycol treatment, electroporation, or particle

bombardment (Marcotte *et al., Nature 335*: 454-457 (1988); McCarty *et al., Cell 66:* 895-905 (1991); Hattori *et al., Genes Dev. 6:* 609-618 (1992); Goff *et al., EMBO J. 9:* 2517-2522 (1990)). Transient expression systems may be used to functionally dissect the regulatory and structural features of expression cassettes comprising operably-linked genetic elements.

Insect Cell Expression

Insect cells may be used as host cells to express recombinant proteins of the present invention (See, e.g., Luckow, V.A., *Protein Eng.* J. L. Cleland., Wiley-Liss, New York, NY: 183-218, 1996, and references cited therein). General methods for expression of foreign genes in insect cells using baculovirus vectors have been described (O'Reilly, D.R., L.K. Miller et al. *Baculovirus Expression Vectors: A Laboratory Manual*. New York, W.H. Freeman and Company, 1992; and King, L.A. and R.D. Possee, *The Baculovirus Expression System: A Laboratory Guide*, London, Chapman & Hall).

A baculovirus expression vector can be constructed by inserting the desired gene (e.g., 11 alpha hydroxylase or oxidoreductase) into a baculovirus transfer vector which can recombine into the baculovirus genome by homologous recombination. Many transfer vectors use a strong baculovirus promoter (such as the polyhedrin promoter) to drive transcription of the desired gene. Some vectors permit the expression of fusion proteins or direct the secretion of proteins from the cell by fusing a eukaryotic secretion signal peptide coding region to the coding region of the desired gene. The plasmid pVL1393 (obtained from Invitrogen Corp., San Diego, California) can be used, for example, to direct transcription of nonfused foreign genes in baculovirus-infected insect cells. The baculovirus transfer vector containing the desired gene is transfected into *Spodoptera frugiperda* (Sf9) insect cells along with circular or linearized genomic baculovirus DNA, and recombinant baculoviruses purified and amplified after one or more plaque assays.

Recombinant baculoviruses can also be created using the baculovirus shuttle vector system (Luckow, V.A. et al., *J. Virol.* **67**(8): 4566-4579, 1993; U.S. Patent 5,348,886) now marketed as the Bac-To-Bac™ Expression System (Life Technologies, Inc., Rockville, MD). The desired genes are inserted downstream from the polyhedrin promoter in mini-Tn7 cassettes that are transposed *in vivo* into a baculovirus shuttle vector genome propagated in *E. coli*. Composite viral DNAs are isolated from *E. coli* and transfected into Sf9 cells and stocks of recombinant baculoviruses are rapidly prepared without the need for multiple

rounds of tedious plaque purification common to methods that rely on homologous recombination.

Recombinant baculoviruses can also created using the Gateway Recombinational Cloning System (Life Technologies) of shuttling genes from vector to vector using modified genetic elements (attachment sites) and modified proteins (e.g., int, IHF, xis) that are involved in the site-specific integration and excision of bacteriophage lambda.

Pure recombinant baculoviruses carrying the 11 alpha hydroxylase or oxidoreductase gene are used to infect cells cultured, for example, in Excell 401 serum-free medium (JRH Biosciences, Lenexa, Kansas) or Sf900-II (Life Technologies). Hydroxylases or oxidoreductases that are localized to membranes can be prepared using standard protocols that fractionate and enrich for enzymes in mitochondrial or microsomal fractions (Engel and White, *Dev Biol. 140*: 196-208, 1990). Hydroxylases or oxidoreductases that are secreted or leak into the medium can also be recovered by standard biochemical approaches.

Simultaneous expression of two or more recombinant proteins in baculovirus-infected insect cells can be carried out by two general approaches. The simplest approach is to coinfect insect cells with titered stocks of recombinant baculoviruses harboring a single heterologous gene under the control of a strong baculovirus promoter, such as the polyhedrin or the p10 promoter. These promoters are highly transcribed during the late stages of infection when most host cell protein synthesis has been shut down. Earlier baculovirus promoters or other insect or eukaryotic cell promoters can also be used to direct synthesis at other times, which generally result in lower expression levels. Varying the ratio of two or more recombinant viruses used in a coinfection or selecting viruses that use different promoters to drive expression of the recombinant protein will permit one skilled in the art to select conditions suitable for optimal expression of the desired recombinant proteins.

Construction of dual- or multiple-expression vectors will also permit the expression of two or more recombinant proteins in baculovirus-infected insect cells. Generally, these vectors permit the introduction two or more gene cassettes into a single locus in the baculovirus genome. The structures of a variety of dual expression vectors have been described (O'Reilly, D. R., L. K. Miller et al. *Baculovirus Expression Vectors: A Laboratory Manual*. New York, W.H. Freeman

and Company, 1992; and King, L. A. and R. D. Possee, *The Baculovirus Expression System: A Laboratory Guide*, London, Chapman & Hall).

Materials and Methods

General methods

General methods of cloning, expressing, and characterizing proteins are found in T. Maniatis, et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, 1982, and references cited therein, incorporated herein by reference; and in J. Sambrook, et al., *Molecular Cloning, A Laboratory Manual,* 2nd edition, Cold Spring Harbor Laboratory, 1989, and references cited therein, incorporated herein by reference. General features and maps of a wide variety of cloning and expression vectors have been also been published (Gacesa, P. and Ramji, D.P., Vectors: Essential Data, John Wiley & Sons, 1994). General methods for the cloning and expression of genes in mammalian cells are also found in Colosimo et al., *Biotechniques* **29**: 314-331, 2000. General and specific conditions and procedures for the construction, manipulation and isolation of polyclonal and monoclonal antibodies are well known in the art (*See*, for example, Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Press, Cold Spring Harbor, New York, 1988).

Unless noted otherwise, all specialty chemicals were obtained from Sigma (St. Louis, MO). Restriction endonucleases and T4 DNA ligase were obtained from Life Technologies (Rockville, MD), New England Biolabs (Beverly, MA), Roche Molecular Biochemicals (Indianapolis, IN), or Promega (Madison, WI). All parts are by weight and temperatures are in degrees centigrade (°C), unless otherwise indicated.

Strains, plasmids, and sequence cross listings

The bacterial strains used in these studies are listed in Table 1. Plasmids used or constructed for this study are listed in Table 2. Brief descriptions of sequences of relevant oligonucleotides, genes, or proteins are listed in Table 3.

Table 1: Strains

Designation	Description or Genotype	Reference/Source
DH5α™	F⁻, *phi*80 d*lac*ZdeltaM15, delta(*lac*ZYA-*arg*F)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17 (rk^-,mk^+), *pho*A, *sup*E44, lambda-, *thi*-1, *gyr*A96, relA1	Life Technologies, Rockville, Maryland
DH10B™	F⁻, *mcr*A D(*mrr-hsd*RMS-*mcr*BC) phi80 d*lac*ZDM15 D*lac*X74 *end*A1 *rec*A1 *deo*R D(*ara*, *leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L	Life Technologies, Rockville, Maryland
DH10Bac™	DH10B harboring the baculovirus shuttle vector bMON14272 (Kan^R) and the helper plasmid pMON7124 (Tet^R)	Life Technologies, Rockville, Maryland; See also Luckow et al., *J. Virol.* 67: 4566-4579 (1993)

Table 2: Plasmids

Plasmid	SEQ ID NO.	Marker	Description	Source
pFastBac1		Amp^R $Gent^R$	Baculovirus donor plasmid containing multiple cloning site downstream of an AcNPV polyhedrin promoter within a mini-Tn7 transposable element capable of being transposed to a baculovirus shuttle vector	Life Technologies Inc. (Rockville, MD); See also Luckow et al., J. Virol. 67: 4566-4579 (1993)
pBluescript II SK		Amp^R	Multifunctional phagemid cloning vector derived from pUC19.	Stratagene, La Jolla, CA
pCRII-TOPO		Amp^R Kan^R	Multifunctional cloning vector for direct cloning of polymerase chain reaction products using the T overhang	Invitrogen, Carlsbad, CA
pSport1		Amp^R	Multifunctional cloning vector for cloning and in vitro transcription from either strand using SP6 or T7 promoters	Life Technologies, Rockville, MD
pGEM-T		Amp^R	A derivative of pGEM-5Zf(+) with single 5' T overhangs at the insertion site to improve the efficiency of PCR product ligation	Promega, Madison, WI
pMON45624	#1	Amp^R $Gent^R$	pFastBac1 *Eco*RI/*Xba*I + PCR fragment *Eco*RI/*Xba*I encoding *Aspergillus ochraceus* 11 alpha hydroxylase	This work

pMON45603		Amp^R	pBluescriptII SK *Bam*HI/HincII + *Bam*HI/HincII 5' segment of human oxidoreductase	This work
pMON45604		Amp^R	pBluescriptII SK *Hinc*II/*Kpn*I + *Hinc*II/*Kpn*I 3' segment of human oxidoreductase	This work
pMON45605	#3	Amp^R $Gent^R$	pFastBac1 *Bam*HI/*Kpn*I + *Bam*HI/*Kpn*I complete coding region of human oxidoreductase cDNA.	This work
pMON45630		Amp^R Kan^R	pCRII-TOPO *Sal*I/*Bam*HI + *Sal*I/*Bam*HI 5' segment of *A. ochraceus* oxidoreductase cDNA	This work
pMON45631		Amp^R Kan^R	pCRII-TOPO *Bam*HI/*Xho*I + *Bam*HI/*Xho*I 3' segment of *A. ochraceus* oxidoreductase cDNA which lacked the intron.	This work
pMON45632	#5	Amp^R $Gent^R$	pFastBac1 *Sal*I/*Xho*I + containing assembled coding region of *Aspergillus ochraceus* oxidoreductase	This work

Table 3: Table of Sequences

SEQ ID NO	Description	Length/Sequence	Type
(SEQ ID NO: 01)	Nucleotide sequence of *Aspergillus ochraceus* 11alphaOH gene from pMON45624	1776	DNA
(SEQ ID NO: 02)	*Aspergillus ochraceus* 11alphaOH protein sequence from pMON45624	514	Protein
(SEQ ID NO: 03)	Nucleotide sequence of human oxidoreductase gene from pMON45605	2031	DNA
(SEQ ID NO: 04)	Human oxidoreductase protein sequence from pMON45605	677	Protein
(SEQ ID NO: 05)	Nucleotide sequence of *Aspergillus ochraceus* oxidoreductase gene from pMON45632	2322	DNA
(SEQ ID NO: 06)	*Aspergillus ochraceus* oxidoreductase protein sequence from pMON45632	705	Protein
(SEQ ID NO: 07)	Primer H. oxred 1A	gatcggatccaatATGGGAGACTCCCACGTGGACAC	DNA
(SEQ ID NO: 08)	Primer H. oxred 1B	CAGCTGGTTGACGAGAGCAGAG	DNA
(SEQ ID NO: 09)	Primer H. oxred 2A	CTCTGCTCTCGTCAACCAGCTG	DNA
(SEQ ID NO: 10)	Primer H. oxred 2B	gatcggtaccttaGCTCCACACGTCCAGGGAGTAG	DNA
(SEQ ID NO: 11)	Primer A.oxred-for1	GACGGIGCIGGTACAATGGA	DNA
(SEQ ID NO: 12)	Primer A.oxred-rev1	TTAIGACCAIACATCITCCTGGTAGC	DNA
(SEQ ID NO: 13)	Primer pSport-for1	CAAGCTCTAATACGACTCACTATAGGGA	DNA
(SEQ ID NO: 14)	Primer A.oxred-rev2	CAGGAACCGATCGACCTCGGAA	DNA
(SEQ ID NO: 15)	Primer A.oxred-rev3	GTCACCCTCACCAGCAGAGCCAATG	DNA
(SEQ ID NO: 16)	Primer A.oxred-rev4	CCACATTGCGAACCATAGCGTTGTAGTG	DNA
(SEQ ID NO: 17)	Primer pSport-for2	GCCAAGCTCTAATACGACTCACTATAGGGAAAGC	DNA
(SEQ ID NO: 18)	Primer A.oxred-for2	gtcgacATGGCGCAACTCGATACTCTC	DNA
(SEQ ID NO: 19)	Primer A.oxred-rev5	ctcgagttaGGACCAGACATCGTCCTGGTAG	DNA
(SEQ ID NO: 20)	Primer A.oxred-for3	GGATCCCTCGCGACCTGTGATCAT	DNA
(SEQ ID NO: 21)	Primer A.oxred-for4	CGAAGATTTCTTGTACAAGGATGAATGGAAGACTTTTC	DNA
(SEQ ID NO: 22)	Primer A.oxred-rev6	CTGAAAAGTCTTCCATTCATCCTTGTACAAGAAATC	DNA
(SEQ ID NO: 23)	11aOH peptide 1	AAAYWLATLQPSDLPELN	Protein
(SEQ ID NO: 24)	11aOH peptide 2	CRQILTPYIHKRKSLKGTTDE	Protein
(SEQ ID NO: 25)	11aOH peptide 3	HMGFGHGVHACPGRFFA	Protein

		SNEI	
(SEQ ID NO: 26)	oxr peptide 1	CTYWAVAKDPYASAGPA MNG	Protein
(SEQ ID NO: 27)	CAA75565; cytochrome P450 monooxygenase [*Gibberella fujikuroi*]		Protein
(SEQ ID NO: 28)	CAB91316; probable cytochrome P450 monooxygenase (lovA) [*Neurospora crassa*]		Protein
(SEQ ID NO: 29)	CAB56503; cytochrome P450 [*Catharanthus roseus*]		Protein
(SEQ ID NO: 30)	AAB94588; CYP71D10p [*Glycine max*]		Protein
(SEQ ID NO: 31)	CAA75566; cytochrome P450 monooxygenase [*Gibberella fujikuroi*]		Protein
(SEQ ID NO: 32)	AAD34552; cytochrome P450 monooxygenase [*Aspergillus terreus*]		Protein
(SEQ ID NO: 33)	CAA75567; cytochrome P450 monooxygenase [*Gibberella fujikuroi*]		Protein
(SEQ ID NO: 34)	CAA76703; cytochrome P450 [*Gibberella fujikuroi*]		Protein
(SEQ ID NO: 35)	CAA57874; unnamed protein product [*Fusarium oxysporum*]		Protein
(SEQ ID NO: 36)	CAA91268; similar to cytochrome P450~cDNA EST yk423b11.3 comes from this gene; [*Caenorhabditis elegans*]		Protein
(SEQ ID NO: 37)	BAA02936 NADPH-cytochrome P450 reductase precursor [*Saccharomyces cerevisiae*]		Protein
(SEQ ID NO: 38)	CAA81550 NADPH cytochrome P450 oxidoreductase [*Aspergillus niger*]		Protein
(SEQ ID NO: 39)	BAA04496 NADPH-cytochrome P450 oxidoreductase [*Mus musculus*]		Protein
(SEQ ID NO: 40)	Universal bacteriophage M13 reverse primer	CAG GAA ACA GCT ATG AC	DNA
(SEQ ID NO: 41)	Universal bacteriophage T7 promoter primer	TAA TAC GAC TCA CTA TAG GG	DNA
(SEQ ID NO: 42)	*Aspergillus ochraceus* Primer 11alphaOH-for	gatcgaattcATGCCCT TCTTCACTGGGCT	DNA
(SEQ ID NO: 43)	*Aspergillus ochraceus* Primer 11alphaOH-rev	gatctctagattacaca gttaaactcgccaTATC GAT	DNA
(SEQ ID NO: 44)	pFastBac1 Primer Bacfwd	CTGTTTTCGTAACAGTT TTG	DNA
(SEQ ID NO: 45)	pFastBac1 Primer PolyA	CCTCTACAAATGTGGTA TG	DNA
(SEQ ID NO: 46)	*Aspergillus ochraceus* Primer 45624-for1	GAGATCAAGATTGCCTT	DNA
(SEQ ID NO: 47)	*Aspergillus ochraceus* Primer 45624-for2	CTTCGACGCTCTCAA	DNA
(SEQ ID NO: 48)	*Aspergillus ochraceus* Primer 45624-rev1	GCAATCTTGATCTCGTT	DNA
(SEQ ID NO: 49)	S90469 human cytochrome P450 reductase [placental, mRNA Partial, 2403 nt].	2403	DNA
(SEQ ID NO: 50)	AAB21814 human cytochrome P450 reductase, placental, partial	676	Protein
(SEQ ID NO: 51)	A60557 human NADPH-ferrihemoprotein reductase	677	Protein
(SEQ ID NO: 52)	P16435 Human NADPH-cytochrome P450 reductase	677	Protein

(SEQ ID NO: 53)	P00389 Rabbit NADPH-cytochrome P450 reductase	679	Protein
(SEQ ID NO: 54)	P00388 Rat NADPH-cytochrome P450 reductase	678	Protein
(SEQ ID NO: 55)	P37040 Mouse NADPH-cytochrome P450 reductase	678	Protein
(SEQ ID NO: 56)	P04175 Pig NADPH-cytochrome P450 reductase	678	Protein
(SEQ ID NO: 57)	Universal bacteriophage SP6 primer	gatttaggtgacactatag	DNA
(SEQ ID NO: 58)	NotI-poly-dT adapter	5' - pGACTAGT TCTAGA TCGCGA GCGGCCGC CC $(T)_{15}$ - 3'	DNA
(SEQ ID NO: 59)	SalI adapter, top strand	5' - TCGACCCACGCGTCCG - 3'	DNA
(SEQ ID NO: 60)	SalI adapter, bottom strand	3' - GGGTGCGCAGGCp - 5'	DNA
(SEQ ID NO: 61)	Primer oxred 1C	GTGGACCACAAGCTCGTACTG	DNA
(SEQ ID NO: 62)	Primer oxred 2C	CATCGACCACCTGTGTGAGCTG	DNA
(SEQ ID NO: 63)	Primer oxred 2D	GTACAGGTAGTCCTCATCCGAG	DNA
(SEQ ID NO: 64)	*Aspergillus niger* NADP CYP450 oxidoreductase Z26838	3710	DNA
(SEQ ID NO: 65)	*Aspergillus niger* NADP CYP450 oxidoreductase CAA81550	693	Protein

Specific Methods

Transformation of E. coli *strains*

E. coli strains such as DH5 alpha and DH10B (Life Technologies, Rockville, MD) are routinely used for transformation of ligation reactions and are the hosts used to prepare plasmid DNA for transfecting mammalian cells. *E. coli* strains, such as DH10B and MON105 (Obukowicz, et al., *Appl. and Envir. Micr.*, 58: 1511-1523, 1992) can be used for expressing the proteins of the present invention in the cytoplasm or periplasmic space.

DH10B and DH5alpha subcloning efficiency cells are purchased as competent cells and are ready for transformation using the manufacturer's protocol. Other *E. coli* strains are rendered competent to take up DNA using a $CaCl_2$ method. Typically, 20 to 50 mL of cells are grown in LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 150 mM NaCl) to a density of approximately 1.0 absorbance unit at 600 nanometers (OD600) as measured by a Baush & Lomb Spectronic spectrophotometer (Rochester, NY). The cells are collected by

centrifugation and resuspended in one-fifth culture volume of $CaCl_2$ solution [50 mM $CaCl_2$, 10 mM Tris-Cl ((10 mM 2-amino-2-(hydroxymethyl) 1,3-propanediol hydrochloride, pH 7.4] and are held at 4°C for 30 minutes. The cells are again collected by centrifugation and resuspended in one-tenth culture volume of $CaCl_2$ solution. Ligated DNA is added to 0.1 ml of these cells, and the samples are held at 4°C for 30-60 minutes. The samples are shifted to 42°C for 45 seconds and 1.0 ml of LB is added prior to shaking the samples at 37°C for one hour. Cells from these samples are spread on plates (LB medium plus 1.5% Bacto-agar) containing either ampicillin (100 micrograms/mL, ug/ml) when selecting for ampicillin-resistant transformants, or spectinomycin (75 ug/ml) when selecting for spectinomycin-resistant transformants. The plates are incubated overnight at 37°C. Colonies are picked and inoculated into LB plus appropriate antibiotic (100 ug/ml ampicillin or 75 ug/ml spectinomycin) and are grown at 37°C while shaking.

DNA isolation and characterization

Plasmid DNA can be isolated by a number of different methods and using commercially available kits known to those skilled in the art. Plasmid DNA is isolated using the Promega Wizard™ Miniprep kit (Madison, WI), the Qiagen QIAwell Plasmid isolation kits (Chatsworth, CA) or Qiagen Plasmid Midi or Mini kit. These kits follow the same general procedure for plasmid DNA isolation. Briefly, cells are pelleted by centrifugation (5000 x g), the plasmid DNA released with sequential NaOH/acid treatment, and cellular debris is removed by centrifugation (10000 x g). The supernatant (containing the plasmid DNA) is loaded onto a column containing a DNA-binding resin, the column is washed, and plasmid DNA eluted. After screening for the colonies with the plasmid of interest, the *E. coli* cells are inoculated into 50-100 ml of LB plus appropriate antibiotic for overnight growth at 37°C in an air incubator while shaking. The purified plasmid DNA is used for DNA sequencing, further restriction enzyme digestion, additional subcloning of DNA fragments and transfection into *E. coli,* mammalian cells, or other cell types.

DNA Sequencing protocols

Purified plasmid DNA is resuspended in dH_2O and its concentration is determined by measuring the absorbance at 260/280 nm in a Baush and Lomb Spectronic 601 UV spectrometer. DNA samples are sequenced using ABI PRISM™ DyeDeoxy™ terminator sequencing chemistry (Applied Biosystems Division of Perkin Elmer Corporation, Lincoln City, CA) kits (Part Number 401388 or 402078)

according to the manufacturer's suggested protocol. Occasionally, 5% DMSO is added to the mixture in repeat experiments, to facilitate the sequencing of difficult templates.

Sequencing reactions are performed in a DNA thermal cycler (Perkin Elmer Corporation, Norwalk, CT) following the recommended amplification conditions. Typically, DNA samples were prepared containing 500 ng of template DNA and 100 ng of primer of choice in thin-walled 0.2 mL PCR tubes that have been brought to 12 uL with Millipore milli-Q (mQ)-quality water. 2 ul of 2 mM Mg^{++} was added to each tube. Tubes were denatured for 5 minutes at 96°C in a Perkin-Elmer System 9700 thermal cycler. After denaturation, the tubes were chilled to a temperature of 4°C by the thermal cycler. 6 ul of ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit was added to each tube. The samples were returned to the thermal cycler and cycle-sequenced using the following program: (1) 96°C for 30 sec; (2) 50°C for 5 sec; (3) 60°C for 4 min, followed by step (1) for 24 additional cycles and then held at 4°C. Cycle sequencing was complete after about 2.5 hours.

Samples are purified to remove excess dye terminators with using Centri-Sep™ spin columns (Princeton Separations, Adelphia, NJ) or purified through a Millipore MAHV N45 50 Multiscreen-HV filtration plate which had been filled with 25 uL Sephadex G-50 superfine resin and 300 uL mQ water. Before loading samples onto filtration plates, the plate was prespun in a centrifuge at 750 x g for 2 min to remove excess water. The samples were loaded onto the resin and the plate spun again at 750 x g for 4 min. The purified sample was collected into a 96-well plate that was placed directly underneath the Sephadex-filled plate during the spin. The liquid in the 96-well plate was dried at room temperature in a Speed Vac. After 45-60 min the DNA was dried and pelleted at the bottom of the plate. Samples were resuspended in 3 uL of a formamide/blue Dextran loading dye and were heated for 2 minutes (see p.33 of Perkin-Elmer Big Dye manual for loading buffer recipe). Samples were loaded onto 48 cm well-to-read length 4.5% acrylamide gels and sequenced for 7 hr using ABI automated DNA sequencers (typically run module Seq Run 48E-1200 and dye set DT, Program BD, Set Any-Primer).

Overlapping DNA sequence fragments are analyzed and assembled into master DNA contigs using Sequencher DNA Analysis software (Gene Codes Corporation, Ann Arbor, MI) or the Perkin-Elmer Data Collection and Sequence Analysis programs to assign bases to the data collected.

BLAST, ClustalW, and Boxshade homology alignment tools

A variety of programs can be used to align nucleotide or peptide sequences to each other and to facilitate homology searches in large sequence databases. BLAST (Basic Local Alignment Search Tool), which implements the statistical matching theory by Karlin and Altschul (*Proc. Natl. Acad. Sci. USA* 87: 2264-2268, 1990; *Proc. Natl. Acad. Sci. USA* 90: 5873-5877, 1993), is a widely used program for rapidly detecting ungapped nucleotide or peptide subsequences that match a given query sequence (Available from the National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov). BLAST uses a heuristic algorithm which seeks local as opposed to global alignments and is therefore able to detect relationships among sequences which share only isolated regions of similarity (Altschul et al., *J. Mol. Biol.* 215: 403-410, 1990).

Two parameters can be varied which alter the sensitivity and quantity of BLAST search results. Parameter B (with a default value of 10) regulates the number of high-scoring segment pairs (alignments) reported in the results. Parameter V (with a default value of 10) is the maximum number of database sequences (hits) for which one-line descriptions will be reported. Matches are based on high-scoring segment pairs (HSPs). Two sequences may share more than one HSP, if the HSPs are separated by gaps. The BLAST algorithm is sensitive to ambiguities in the sequence and is not well-suited for sequences that contain many gaps.

The program blastp compares an amino acid query sequence against a protein sequence database. blastn compares a nucleotide query sequence against a nucleotide sequence database. blastx compares a nucleotide query sequence translated in all reading frames against a protein sequence database. You could use this option to find potential translation products of an unknown nucleotide sequence. tblastn compares a protein query sequence against a nucleotide sequence database dynamically translated in all reading frames. tblastx compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database (See http://www.ncbi.nlm.nih.gov/ Education/BLASTinfo/ for more information on BLAST, related programs, and pattern matching algorithms).

Nucleotides searches performed with BLAST, score = 98-557, word length 514 letters, were used to obtain nucleotide sequences homologous to nucleic acid molecules of the present invention. Protein searches are performed with BLASTP,

score = 50, word length = 3 to obtain amino acid sequences homologous to a reference polypeptide (e.g., SEQ ID NO: 2).

Clustal W version 1.74, which implements a different algorithm for alignment of multiple DNA or protein sequences, was also used to prepare alignments and to assign percent identities between different sequences. This program improves the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice (Thompson et al., *Nucleic Acids Research*, 22(22):4673-4680, 1994). The default parameters for version 1.74 were used facilitate alignments and to assign percent identities between two sequences. The input consisted of sequences in FASTA format and the output is the alignment shown in the figures. For nucleic acid sequences, the iub DNA weight matrix was used. For amino acid sequences, the blosum protein weight matrix was used (See http://www.ncbi.nlm.nih.gov/Education/BLASTinfo/ for more information on BLAST, related programs, and pattern matching algorithms.

Boxshade v 3.31 is a public domain program for creating nicely formatted printouts from muliple-aligned protein or DNA sequences. Boxshade, by itself, does not create alignments, but applies shading or coloring to files that were previously prepared by other sequence alignment programs. The inputs to Boxshade are the alignments created by ClustalW and the threashold values for the residues to be colored or shaded. In most cases, except where specified, a 50% identity value was used. With this setting, if a position has greater than or equal to half of the sequences sharing an identical residue, then it is shaded. Boxshade is available by ftp from ftp. or by e-mail from Kay Hofmann (khofmann@isrec-sun1-unil.ch or Michael D. Baron (michael.baron@bbsrc.ac.uk).

Protein Purification and Characterization

Protein purification can be accomplished using any of a variety of chromatographic methods such as: ion exchange, gel filtration or hydrophobic chromatography or reversed phase HPLC. In some cases, proteins which are properly folded can be affinity-purified using affinity reagents, such as monoclonal antibodies or receptor subunits attached to a suitable matrix. These and other protein purification methods are described in detail in Methods in Enzymology, Volume 182 "Guide to Protein Purification" edited by Murray Deutscher, Academic Press, San Diego, California, 1990.

The purified protein can be analyzed by RP-HPLC, electrospray mass spectrometry, and SDS-PAGE. Protein quantitation is done by amino acid composition, RP-HPLC, and/or Bradford protein dye-binding assays. In some cases, tryptic peptide mapping is performed in conjunction with electrospray mass spectrometry to confirm the identity of the protein.

Examples

The following examples will illustrate the invention in greater detail, although it will be understood that the invention is not limited to these specific examples. Various other examples will be apparent to the person skilled in the art after reading the present disclosure without departing from the spirit and scope of the invention. It is intended that all such other examples be included within the scope of the appended claims.

Example 1 - Preparation of *A. ochraceus* spores for RNA extraction

Aspergillus ochraceus ATCC 18500 stock culture (50 ul) was grown for 3-4 days on plates containing sporulation medium: 50 g/L molasses, 5 g/L cornsteep liquid, 5 g/L KH_2PO_4, 25 g/L NaCl, 25 g/L glucose, 20 g/L agar, and 0.4 g/L progesterone, pH 5.8. Progesterone was included in the media to induce the steroid 11 α-hydroxylase. Spores were scraped from the plates into 5 to 7 ml saline, washed in saline, collected by centrifugation, and suspended in saline containing 15% glycerol. The spores were frozen on dry ice and stored at -80°C. Approximately 0.8 g spores were incubated at 30°C in a 1 liter flask containing 400 ml 1% glucose, 50 mM KH_2PO_4 and 0.1 g canrenone, pH 7.0. This treatment prior to spore disruption has three benefits: (1) to induce the steroid 11 α-hydroxylase by incubation with canrenone; (2) to determine whether the spores were catalyzing the 11 α-hydroxylation of canrenone; (3) and to soften the spore wall. After approximately 26 hours of incubating with shaking at 30°C to provide better aeration, the spores were collected by centrifugation. Visual inspection with the aid of a microscope indicated that very few had started to germinate. The spore pellets were flash frozen in liquid nitrogen and stored at -80°C. The media was analyzed for presence of 11 alpha hydroxy canrenone by HPLC to determine whether spores used for library construction demonstrated the desired activity.

Example 2 - *A. ochraceus* spores catalyze 11 α-hydroxylation of canrenone

Approximately 160 ml of media from the spore induction was extracted three times with 70 ml ethyl acetate to collect the steroid substrate and products. The organic phase was dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. The residue was dissolved in 8 ml methanol so that the final concentration of canrenone was approximately 15 mM (assuming quantitative recovery). The media extract was diluted 10- to 15-fold into 50% methanol for HPLC analysis. Stock solutions of canrenone and 11 α-hydroxy canrenone were prepared in methanol. Standards for HPLC analysis were prepared from these stock solutions by diluting to a final concentration of 750 uM with 50% methanol. Media extract and standards were chromatographed over a C-4 reverse phase HPLC column. The media exhibited a component with the same retention time as the 11 α-hydroxy canrenone standard, as monitored at 254 nm (data not shown).

Example 3 - Growth of *A. ochraceus* Mycelia for RNA extraction

Liquid cultures of *Aspergillus ochraceus* mycelia were grown in 10 g/L peptone, 10 g/L yeast extract and 10 g/L glucose containing 20 g/L canrenone for 24 to 72 hours at 28°C in a volume of 160 ml. Ten ml samples of cells were filtered, washed with cold water, frozen, and stored at -80°C.

Example 4 - Extraction of total RNA from induced spores

Approximately 0.4 g spores were disrupted in 40 ml Trizol reagent (Life Technologies, Rockville, MD) using a Mini-Beadbeater™ model 3110 (Biospec Products, Bartlesville, OK). Briefly, spore-Trizol mixture was subjected to four 30 second pulses at low speed. Between pulses, tubes containing spores were chilled on ice. Visual inspection with the aid of a microscope indicated that the majority of the spores were disrupted by this treatment. The debris was pelleted by low-speed centrifugation and the total RNA in the supernatant was extracted following the manufacturer's recommended protocols for use with Trizol. Briefly, 2 ml chloroform was added for each 10 ml Trizol in 11 ml polypropylene centrifuge tubes. Following a 3 minute extraction of proteins, phase separation was done by centrifugation and the aqueous phase containing the RNA was transferred to a clean tube for precipitation with an equal volume of isopropanol. The precipitated RNA was recovered by centrifugation and washed with 70% ethanol. The RNA was resuspended in 10 ml water, re-extracted with chloroform and precipitated with ethanol overnight at -20°C. Total RNA (3 mg) was recovered by

centrifugation and rehydrated in 2 ml water, and precipitated on ice by adding an equal volume of cold 4 M lithium chloride. This precipitation was done to remove DNA, carbohydrates, heme, and other impurities which can carry over from guanidine methods. The RNA was recovered by a 25 minute centrifugation.

Example 5 - Extraction of total RNA from induced mycelia

Approximately 0.5 g wet weight cells were pulverized to a fine powder under liquid nitrogen with a mortar and pestle pre-chilled in dry ice. The powder was added to 10 ml Trizol Reagent (Life Technologies) and homogenized with a Kinematica polytron (Kinematica AG, Lucerne, Switzerland) at setting #4. Cellular debris was removed by centrifugation prior to chloroform extraction. The aqueous phase containing nucleic acids was precipitated with isopropanol for 10 minutes at room temperature. The precipitate was collected by centrifugation and washed with 70% ethanol. The RNA was rehydrated in water and re-extracted with chloroform to remove any residual proteins. The aqueous phase was precipitated at -20°C with 1/10 volume of 3 M sodium acetate and 2.5 volumes absolute ethanol. The final yield was 424 ug. Approximately 4 ug and 16 ug of total RNA were separated by electrophoresis through a 1.2% agarose gel and visualized by staining in ethidium bromide. Chromosomal DNA was present as a minor contaminant.

Example 6 - Extraction of Total RNA from HepG2 cells

Hepatocellular human liver carcinoma cells (HepG2), ATCC HB-8065, were maintained in DMEM high glucose media supplemented with Penstrep, glutamate and 10% fetal bovine serum (Life Technologies, Rockville, MD). Cells were induced overnight with 0.05% ethanol and harvested for RNA extraction by trypsinization. Briefly, the cell pellet was resuspended in >10X volumes of 4 M guanidine isothiocyanate, 50 mM Tris-HCl, pH 7.5, 25 mM EDTA (solution D, Life Technologies) and then vortexed. Water and sodium acetate, pH 4.1, were added such that the final concentration of sodium acetate was 0.1 M. The RNA solution was extracted with one half volume of chloroform and placed on ice for 15 minutes. The aqueous phase was re-extracted with chloroform and precipitated overnight with isopropanol. Total RNA was resuspended in solution D and re-precipitated with isopropanol, followed by two precipitations in water containing 0.3 M sodium acetate pH 5.5 and 2.5 volumes of ethanol. PolyA$^+$ selection was performed twice as described below.

Example 7 - PolyA$^+$ Selection of mRNA

PolyA$^+$ RNA was selected from total RNA with an Eppendorf 5Prime, Inc. kit (Boulder CO). Briefly, each 1 mg of total RNA was selected twice over a column containing oligo dT cellulose. The column slurry was packed by gentle centrifugation and equilbrated with 0.5 M NaCl. RNA was allowed to bind to the dT cellulose for 15 minutes at room temperature. The columns were washed once with 0.5 M NaCl, and twice with 0.1 M NaCl. PolyA$^+$ RNA was eluted in 0.5 ml 10 mM Tris-HCl, 1 mM EDTA, pH 7.5. The selection by oligo dT cellulose was performed twice. The mRNA was precipitated at -20°C with 0.3 M sodium acetate in 50% ethanol, with glycogen added as carrier.

Example 8 - cDNA Synthesis and Library Construction

The Superscript™ Plasmid System for cDNA Synthesis and Plasmid Cloning kit (Life Technologies) was used for cDNA systhesis and library construction. Superscript II reverse transcriptase catalyzed the first strand of cDNA in a 20 ul reaction for 1 hour at 42°C. The final composition was 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 50 uM each dATP, dCTP, dGTP and dTTP, 50 ug/ml oligo-dT-*NotI* primer-adaptors that were phosphorylated at their 5' end (Life Technologies) and 50,000 units/ml Superscript II reverse transcriptase.

oligo-dT-NotI primer-adapter
5' - pGACTAGT TCTAGA TCGCGA GCGGCCGC CC $(T)_{15}$ - 3' (SEQ ID NO: 58)
*Spe*I *Xba*I *Nru*I *Not*I

A radiolabeled tracer ([α-^{32}P]dCTP) was not added. The second strand of cDNA was synthesized in a reaction volume of 150 ul. The final composition of this mixture including the first strand reaction was 25 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM $(NH_4)_2SO_4$, 0.15 mM B-NAD$^+$, 250 uM each dATP, dCTP, dGTP and dTTP, 1.2 mM DTT, 65 units/ml *E. coli* DNA ligase, 250 units/ml *E. coli* DNA polymerase I and 13 units/ml *E. coli* Rnase H. After a 2 hour incubation at 16°C, 10 units of T4 DNA polymerase was added, and incubated 5 minutes at 16°C. The reaction was stopped with 10 ul 0.5 M EDTA and the cDNA was separated from cDNAs smaller than 300 base pairs, primer-adaptors and deoxynucleotides with GENECLEAN II (BIO 101 Inc. La Jolla, CA). Annealed *Sal I* adaptors (Life Technologies) that were phosphorylated at their 5' blunt end were ligated to the cDNA overnight at 16°C.

*Sal*I adapter

5' - TCGACCCACGCGTCCG - 3' (SEQ ID NO: 59)
3' - GGGTGCGCAGGCp - 5' (SEQ ID NO: 60)

GENECLEAN II was used to remove the adaptors. The cDNA was then digested with *NotI*. QIAquick columns (QIAGEN, Valencia, CA) were used to remove small DNA fragments from the cDNA, which was ethanol precipitated.

Example 9 - Size Fractionation of cDNA

The cDNA was enriched for species approximately 1.5 kb and larger by gel electrophoresis through 0.8% Sea-Plaque agarose (FMC BioProducts, Rockland ME) in TAE buffer. The preparative gel had a lane of DNA size markers which was excised from the gel after electrophoresis and stained with ethidum bromide for visualization under ultraviolet light next to a ruler so that the appropriate region of the cDNA could be recovered from the gel. GENECLEAN II was used to extract the cDNA, which was eluted in 20 ul water.

Example 10 - Library Construction in Vector pSport1 and Electroporation into E. coli

An aliquot of the size-selected cDNA was ligated overnight at 4°C with pSport1 (Life Technologies, Inc., Rockville, MD) predigested with *NotI* and *SalI* in a 20 ul reaction containing 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 5% (w/v) PEG 8000, 1 mM DTT, 2.5 ug/ml pSport1, approximately 0.5 ug/ml cDNA, and 50 units/ml T4 DNA ligase. The ligation mixture was precipitated by the addition of 12.5 ul 7.5 M ammonium acetate, 5 ul yeast tRNA carrier and 70 ul absolute ethanol. The ligated cDNA was recovered by centrifugation at room temperature for 20 minutes and rehydrated in 5 ul sterile water. One ul of the ligated cDNA was introduced into ElectroMAX DH10B *E. coli* (Life Technologies) by electroporation. Cells were allowed to recover in 1 ml SOC medium (Life Technologies) for 1 hour at 37°C, before plating an aliquot on LB with 100 ug/ml ampicillin. The titer of the *Aspergillus ochraceus* spore library (designated LIB3025) was determined by preparing serial dilutions of the cell suspension in SOC. The equivalent of 1 ul, 0.1 ul and 0.01 ul samples of the cell suspension were plated, and the resulting titer was calculated to be 1.75 x 10^6/ml colony forming units.

Example 11 - Identification of clones encoding cytochrome P450 enzymes by DNA sequence analysis and construction of plasmid pMON45624 encoding *Aspergillus ochraceus* 11 alpha hydroxylase

Cloning of 11 alpha hydroxylase from Aspergillus ochraceus

Approximately 2,000 colonies were selected on LB agar plates containing 100 ug/ml ampicillin and miniprep plasmid DNA samples were prepared for sequencing. Unidirectional sequencing was performed from the 3' end of the expressed sequence tags (ESTs) beginning at the *NotI* site encompassing part of the poly dT primer used for cDNA synthesis. Two universal primers were used to facilitate the sequencing:

M13 reverse: CAG GAA ACA GCT ATG AC (SEQ ID NO: 40)
T7 promoter: TAA TAC GAC TCA CTA TAG GG (SEQ ID NO: 41)

Most known cytochrome p450s contain a conserved heme-binding region approximately 50 amino acid residues (150 nucleotides) upstream of the stop codon (Nelson *et al*, *Pharmacogenetics* **6**: 1-42, 1996). The 2,000 ESTs were screened for sequences encoding the canonical heme-binding motif (FXXGXXXCXG, where "X" is any amino acid) in the appropriate region using BLASTX and visual inspection of the sequences scored as hydroxylases for the canonical heme-binding motif. Only fifteen ESTs had the heme-binding motif. One EST was unique and the other fourteen appeared to be overlapping sequences. The cDNA inserts from seven clones encoding putative cytochrome p450 enzymes were then sequenced to completion. All seven encoded the same enzyme.

Gene Amplification of Aspergillus ochraceus *11 alpha hydroxylase*

The coding region of the 11 alpha hydroxylase was amplified by PCR using a unique clone from the *A. ochraceus* cDNA spore library (LIB3025) as a template. The primers included recognition sites for *Eco*RI (forward) and *Xba*I (reverse) for directional cloning into pFastbac1. Amplification was carried out for 32 cycles using a PCR core kit (Roche) and 50 pmol of each primer. One cycle consisted of a denaturation step at 94°C for 45 seconds, an annealing step at 60°C for 45 seconds, and an elongation step at 72°C for 60 seconds.

Primer 11alphaOH-for: gatcgaattcATGCCCTTCTTCACTGGGCT (SEQ ID NO: 42)
Primer 11alphaOH-rev: gatctctagaTTACACAGTTAAACTCGCCATATCGAT (SEQ ID NO: 43)

Construction of pMON45624

The amplified fragments described above were purified through a QIAquick column (Qiagen, Valencia CA) and digested with *Eco*RI and *Xba*I prior to ligation into pFastBac1 cleaved with *Eco*RI and *Xba*I. The resulting plasmid was designated pMON45624 and the DNA sequence verified using primers based on the vector sequence and internal primers based on the 11 alpha hydroxylase sequence (shown below).

Primer Bacfwd:	CTGTTTTCGTAACAGTTTTG	(SEQ ID NO: 44)
Primer PolyA:	CCTCTACAAATGTGGTATG	(SEQ ID NO: 45)
Primer 45624-for1:	GAGATCAAGATTGCCTT	(SEQ ID NO: 46)
Primer 45624-for2:	CTTCGACGCTCTCAA	(SEQ ID NO: 47)
Primer 45624-rev1:	GCAATCTTGATCTCGTT	(SEQ ID NO: 48)

The nucleotide and predicted amino acid sequences of the cloned 11 alpha hydroxylase are displayed in Figure 1 as SEQ ID NO: 1 and SEQ ID NO: 2, respectively.

Figure 4 sets forth an amino acid homology alignment of *A. ochraceus* 11 alpha hydroxylase cloned in pMON45624 and aligned with related enzymes found in GenBank using BLAST. Figure 5 is a phylogenetic tree showing the this relationship graphically. Figure 6 shows the percent homology between *Aspergillus ochraceus* steroid 11 alpha hydroxylase and the top 10 enzymes found in GenBank using BLAST, calculated using Clustal W and Boxshade.

Example 12 - Amplification of cDNA encoding human NADPH Cytochrome P450 reductase and cloning into plasmids pMON45603, pMON45604, and pMON45605

Gene Amplification of human oxidoreductase

Approximately 1 ug polyA$^+$ mRNA from HepG2 cells was heated to 65°C for 10 minutes with 100 ng random hexamers (Invitrogen, Carlsbad, CA) in an 11 ul reaction. The mixture was chilled on ice, then incubated at 42°C for 75 minutes in a 20 ul reaction containing 1 ul RNase inhibitor (Promega, Madison, WI), 0.01 M DTT, 5 mM dNTPs, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 1 ul SuperScriptII enzyme (Life Technologies). The reverse transcriptase was inactivated by heating to 95°C for 2 minutes. First strand cDNA was stored at -20°C. Forward and reverse primers were based on the nucleotide sequence of accession number S90469 (human placental partial mRNA encoding cytochrome P450 reductase (SEQ ID NO: 49)). The accession number of the corresponding

protein sequence is AAB21814 (SEQ ID NO: 50). The human oxidoreductase was cloned in two pieces which were assembled in pFastBac1 (Life Technologies) by ligation at an internal *Hinc*II site. The primers included restriction sites for directional subcloning into pFastBac1.

```
Primer H. oxred 1A: gatcggatccaatATGGGAGACTCCCACGTGGACAC   (SEQ ID NO: 07)
Primer H. oxred 1B: CAGCTGGTTGACGAGAGCAGAG                 (SEQ ID NO: 08)
Primer H. oxred 2A: CTCTGCTCTCGTCAACCAGCTG                 (SEQ ID NO: 09)
Primer H. oxred 2B: gatcggtaccttaGCTCCACACGTCCAGGGAGTAG    (SEQ ID NO: 10)
```

The second strand was synthesized using 400 uM dNTP and 167 nM of each primer set per 150 ul reaction. Amplification was performed with Deep Vent polymerase (New England Biolabs, Beverly, MA). The reaction for segment 2 (the 3' half of the oxidoreductase cDNA) was adjusted to 5% DMSO. The amplification included an initial cycle of denaturation at 94°C for 90 seconds, followed by annealing at 62°C for 2 minutes and elongation at 72°C for 2 minutes. This was followed by 30 cycles, consisting of a 45 second denaturation step, a 45 second annealing step, and a 60 second elongation step. The elongation step was extended to 5 minutes for the final cycle.

Construction of pMON45603, pMON45604, pMON45605

The PCR fragments for the 5' half of the oxidoreductase cDNA were digested with *Bam*HI and *Hinc*II. The PCR fragments for the 3' half of the oxidoreductase cDNA were digested with *Hinc*II and *Kpn*I and ligated into pBluescript II (Stratagene, La Jolla, CA) for sequencing. The resulting plasmids were designated pMON45603 (5' segment) and pMON45604 (3' segment). The *Bam*HI/*Hinc*II fragment from pMON45603 and the *Hinc*II/*Kpn*I fragment from pMON45604 were ligated into pFastbac1 cut with *Bam*HI and *Kpn*I, to generate pMON45605.

Sequencing primers were based on the sequence of GenBank accession number S90469 (SEQ ID NO 49), a cDNA encoding cytochrome P450 reductase [human, placenta, mRNA Partial, 2403 nt]. The cognate protein sequence is: AAB21814 (SEQ ID NO 50) cytochrome P450 reductase {EC 1.6.2.4} [human, placenta, Peptide Partial, 676 aa] [Homo sapiens]. The cDNA insert of pMON45603 was sequenced using primer oxred 1C, and the cDNA insert of pMON45604 was sequenced using primer oxred 2C and 2D. Universal T7 (SEQ ID NO: 41) and M13 reverse (SEQ ID NO: 40) primers, which annealed to vector sequences flanking the cDNA inserts were also used for sequencing.

```
Primer oxred 1C: GTGGACCACAAGCTCGTACTG   (SEQ ID NO: 61)
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Primer oxred 2C: CATCGACCACCTGTGTGAGCTG (SEQ ID NO: 62)
Primer oxred 2D: GTACAGGTAGTCCTCATCCGAG (SEQ ID NO: 63)

The nucleotide and predicted amino acid sequences of the cloned human oxidoreductase are displayed in Figure 2 as SEQ ID NO: 3 and SEQ ID NO: 4, respectively. Figure 11 sets forth an alignment of human oxidoreductase with top 4 hits from SwissProt. Figure 12 sets forth a phylogenetic tree displaying the genetic relatedness of human oxidoreductase, to these hits. Figure 13 shows the percent identity between human oxidoreductase and top 4 hits from SwissProt.

Example 13 - Amplification of cDNA encoding NADPH cytochrome P450 reductase from *A. ochraceus* and cloning into plasmids pMON45630, pMON45631, and pMON45632.

Gene Amplification of Aspergillus ochraceus *oxidoreductase*

An alignment of sequences from the *Aspergillus niger* cprA gene accession number Z26938 (SEQ ID NO: 65) and a partial cDNA clone 804561639F1 from *Aspergillus fumigatus* (PathoSeq Database, Incyte Pharmaceuticals) was visually scanned to select regions of high homology for the design of primers for PCR. A primer set was selected which spanned the coding region of the cprA gene product from amino acids 203 to 693.

Primers were selected from the 5' most region of overlap where the amino acid sequence was identical between both and the nucleic acid sequence differed by 2 positions in the 3rd codon position. For the 3' primer, the nucleic acid encoding the stop codon, last 7 amino acid residues and 2 additional bases corresponding to second and third positions in the codon of the amino acid residue 8 positions from the stop codon encodes ARG in A. *niger* and SER in *A. fumigatis* (CGC vs. AGC). Inosines replaced the third base in codons when there was a discrepancy between the *A. niger* and *A. fumigatus* sequence.

Primer A.oxred-for1: GACGGIGCIGGTACAATGGA (SEQ ID NO: 11)
Primer A.oxred-rev1: TTAIGACCAIACATCITCCTGGTAGC (SEQ ID NO: 12)
(where I = Inosine)

A partial cDNA clone was amplified from approximately 5 ug of total RNA extracted from *A. ochraceus* mycelia. Before the first strand synthesis, the RNA was heated to 65°C for 10 minutes with 100 ng random hexamers (Promega Madison WI) in an 11 ul reaction mixture. The mixture was chilled on ice, then incubated at 42°C for 75 minutes in a 20 ul reaction containing 1 ul RNase inhibitor (Promega), 0.01 M DTT, 5 mM dNTPs, 50 mM Tris-HCl, pH 8.3), 75 mM

KCl, 3 mM $MgCl_2$ and 1 ul SuperScriptII (LTI). The reverse transcriptase was inactivated by heating to 95°C for 2 minutes. The first strand cDNA was stored at -20°C. The second strand was synthesized using 5 ul of the first strand as template. The reaction included 500 nM primers, 200 uM each dNTP, and Taq polymerase and buffer as supplied in PCR core kit (Roche Molecular Biochemicals, Indianapolis, IN). Amplification was performed using 32 cycles of a 30 second denaturation step at 94°C, a 30 second annealing step at 60°C and a 60 second elongation step at 72°C. The amplified DNA products were cloned into pGEM-T (Promega, Madison, WI) and sequenced using universal T7 (SEQ ID NO: 41) and SP6 (SEQ ID NO: 57) primers.

Primer SP6 GATTTAGGTGACACTATAG (SEQ ID NO: 57)

Alignment of the sequences with the *A. niger* cprA gene revealed that the *A. ochraceus* clones had an intron in the same position as the intron in the *A. niger* gene. This indicated that the *A. ochraceus* PCR products might have been amplified from a genomic DNA contaminant of the total RNA. A reverse primer based on the *A. ochraceus* sequence was designed to amplify the approximately 600 missing bp including the initial methionine. The *A. ochraceus* cDNA library was then used as a template for PCR. The forward primer was based on the reverse complement of vector pSport1 (Life Technologies) bases 299 to 326. The other primer, A.oxred-rev2 was bases on the *A. ochraceus* sequence encoding residues 326-333.

Primer pSport-for1: CAAGCTCTAATACGACTCACTATAGGGA (SEQ ID NO: 13)
Primer A.oxred-rev2: CAGGAACCGATCGACCTCGGAA (SEQ ID NO: 14)

The *A. ochraceus* spore library size made from gel-purified fragments >1.5 kb in size was then used as a template for amplifying the final 200 bases of coding region. Two new reverse primers were designed from the A.oxred sequence, and a new forward primer based on pSport1 (bases 295-328) was also used.

Primer A.oxred-rev3: GTCACCCTCACCAGCAGAGCCAATG (SEQ ID NO: 15)
Primer A.oxred-rev4: CCACATTGCGAACCATAGCGTTGTAGTG (SEQ ID NO: 16)
Primer pSport-for2: GCCAAGCTCTAATACGACTCACTATAGGGAAAGC (SEQ ID NO: 17)

Amplification was performed using an Elongase polymerase kit (Life Technologies, Rockville MD) for 35 cycles consisting of a denaturation step at 94°C for 30 seconds, an annealing step at 63°C for 30 seconds, and an elongation step at 68°C for 5 minutes. The PCR products were cloned directly into pCRII TOPO (Invitrogen). Twelve clones were sequenced, and the composite sequence, extended

for 232 bases upstream of the initial methionine, and included 2 in-frame stop codons (Data not shown).

Primers incorporating the complete coding region of A.oxred were designed with a 5' *Sal*I site and a 3' *Xho*I site for ligation into expression vector pFastBac1.

Primer A.oxred-for2: gtcgacATGGCGCAACTCGATACTCTC (SEQ ID NO: 18)
Primer A.oxred-rev5: ctcgagttaGGACCAGACATCGTCCTGGTAG (SEQ ID NO: 19)

A. ochraceus total RNA was used as a template for PCR with these primers and the Elongase kit. Amplification consisted of 35 cycles with a 30 second denaturation step at 94°C, a 30 second annealing step at 64°C, and a 5 minute elongation step at 68°C. An aliquot of the cDNA from reaction ran as a single band of approximately 2.1 kb.

Construction of pMON45630

The PCR products were cloned directly into pCRII-TOPO (Invitrogen, Carlsbad, CA). All clones contained the internal intron noted earlier. One clone was designated pMON45630.

Construction of pMON45631 and pMON45632

A strategy based on two step PCR from an internal *Bam*HI site approximately 170 bp upstream of the 5' splice site was employed to generate clones lacking the intron.

Primer A.oxred-for3: GGATCCCTCGCGACCTGTGATCAT (SEQ ID NO: 20)
Primer A.oxred-for4: CGAAGATTTCTTGTACAAGGATGAATGGAAGACTTTTC (SEQ ID NO: 21)
Primer A.oxred-rev6: CTGAAAAGTCTTCCATTCATCCTTGTACAAGAAATC (SEQ ID NO: 22)

Primers A.oxred-for4 and rev6 were complementary and flanked the intron. The first PCR reaction used an A.oxred clone linearized at the internal *Bam*HI site as template. Polymerase and buffers were supplied by the PCR core kit (Roche Molecular Biochemicals, Indianapolis, IN). Primer and dNTP concentrations were 500 nM and 200 uM, respectively. Two reactions were performed, using a combination of A.oxred-for3 with A.oxred-rev6, and A.oxred-for4 with A.oxred-rev5. Following a 2 minute initial denaturation, 28 cycles of PCR amplification were performed. One cycle included a 45 second denaturation at 94°C, a 45 second denaturation step at 62°C and a 45 second elongation step at 72°C. One ul of each reaction served as template for the second PCR amplification with primers A.oxred-for3 and A.oxred-rev5 using Elongase enzyme and buffers. Amplification

consisted of 30 cycles with a 30 second denaturation step at 94°C, a 30 second annealing step at 62°C, and a 5 minute elongation step at 68°C. The PCR products were directly cloned into pCRII-TOPO. DNA sequencing demonstrated that the intron had been removed. This clone was designated pMON45631.

Plasmid pMON45632 was constructed in a three-way ligation by ligating the *SalI/Bam*HI fragment from pMON45630 with the *Bam*HI/*Xho*I fragment from pMON45631 and vector pFastBac1, which had been cut with *Sal*I and *Xho*I and de-phosphorylated to enhance the recovery of vectors with the desired inserts.

The nucleotide and amino acid sequences of the cloned *Aspergillus ochraceus* 11 oxidoreductase are displayed in Figure 3 as SEQ ID NO: 5 and SEQ ID NO: 6, respectively. Figure 7 sets forth the amino acid homology of *Aspergillus ochraceus* and human oxidoreductase to NADPH cytochrome P450 reductases from *A. niger*, mouse, and *S. cerevisiae.* Figure 8 sets forth the amino acid alignment for *A. ochraceus, A. niger,* and *S. cerevisiae* oxidoreductases. Figure 9 is a phylogenetic tree showing the relatedness of *Aspergillus ochraceus* and human oxidoreductase to reductases from *A. niger*, yeast, and mouse. Figure 10 shows the percent homology between *Aspergillus ochraceus* steroid 11 alpha hydroxylase and the oxidoreductases from *A. niger*, yeast, and mouse, calculated using Clustal W and Boxshade.

Example 15: Generation of polyclonal antibodies recognizing *Aspergillus ochraceus* 11 alpha hydroxylase and *Aspergillus ochraceus* NADPH cytochrome p450 reductase

Generation of anti-11-a-hydroxylase Antibodies

Polyclonal antibodies against *Aspergillus ochraceus* 11 alpha hydroxylase and NADPH cytochrome p450 reductase were raised in rabbits against synthetic peptides (prepared by Sigma/Genosis, The Woodlands, TX) corresponding to several regions of the following predicted protein sequences:

```
11aOH peptide 1:   AAAYWLATLQPSDLPELN      (SEQ ID NO: 23)
11aOH peptide 2:   CRQILTPYIHKRKSLKGTTD    (SEQ ID NO: 24)
11aOH peptide 3:   HMGFGHGVHACPGRFFASNEI   (SEQ ID NO: 25)
oxr peptide 1:     CTYWAVAKDPYASAGPAMNG    (SEQ ID NO: 26)
```

The 11aOH peptide 2 (SEQ ID NO: 24) corresponds to the G helix, G/H loop, and H helix region present in an alignment of the amino acid sequence of 11 alpha hydroxylase with the corresponding sequence of CYP3A4 described by Wang and

Lu, (*Drug Metab. Dispos.* 25(6), 762-767, 1997). The 11aOH peptide 3 (SEQ ID NO: 25) corresponded to the peptide fragment from the heme-binding domain.

Immunological grade peptides were monitored for purity using reverse phase high performance liquid chromatography (HPLC). Each peptide was conjugated to keyhole limpet hemacyanin (KLH) and suspended in Complete Freund's Adjuvant. The conjugated peptide was then injected subcutaneously at multiple sites into rabbits. Each conjugated peptide was injected into two rabbits. All subsequent immunizations were given in incomplete Freund's Adjuvant. In general, five subsequent injections were given at two-week intervals following the initial immunization. IgG fractions were affinity-purified using a Sepharose-Protein A column. Fractions from the two rabbits injected with each peptide were combined at a 1:1 ratio. The pooled anti-11 alpha hydroxylase (rabbits GN 1187/1188) was 0.34 mg/ml IgG. The pooled anti-oxred (rabbits GN 2023/2024) was 0.26 mg/ml IgG. The combined IgGs were each diluted 1:10, 1:100 and 1:1,000 for a pilot experiment to determine which was dilution was optimal for probing Western blots. The 1:10 dilution gave the best results and was used for probing subsequent Westerns.

Example 16 - Insect Cell Infection and Heterologous Expression

Proteins were expressed in *Sf*9 insect cells using baculovirus shuttle vectors (Luckow et al., *J. Virol.* **67**: 4566-4579, 1993). The baculovirus shuttle vector (bacmid) contains a mini-F replicon for expression in bacterial cells, a kanamycin resistance marker for selection, and *att*Tn7 (the target site for the bacterial Tn7 transposon) within the *lacZα* sequence. Each of these elements is inserted into the polyhedrin locus of the *Autographa californica* nuclear polyhedrosis virus (AcNPV, the native baculovirus) genome. A donor plasmid (pFastBac1, Life Technologies) was used to deliver the gene to be expressed and was inserted into the bacmid via the bacterial Tn7 transposition elements. pFastBac1 contains the Tn7 left and right ends flanking the polyhedrin promoter, a polylinker cloning sequence, the SV40 polyA transcription termination sequence, and the gentamicin resistance gene for selection. Recombinant viruses were generated following transformation of the pFastBac1 plasmid, which contained a single 11 alpha hydroxylase or oxidoreductase cDNA, into DH10Bac *E. coli* cells (Life Technologies) that contained the bacmid and helper plasmid.

Transfections were performed using CellFectin™ reagent (Life Technologies) following the manufacturer's protocol for *Spodoptera frugigperda*

(Sf9) cells. Cells were seeded in 6-well tissue culture plates at 9 x 10^5 cells per well in SF-900 serum-free medium (Life Technologies) and allowed to attach for at least one hour. The transfection mixtures were made following the addition of 5 ul miniprep DNA and 5µl Cellfectin to polystyrene tubes that contained 200 ul SF-900 medum. The mixtures were allowed to incubate for 15-30 minutes at room temperature. Prior to transfection, 800 ul SF-900 medium was added to each tube. The cells were washed one time with 2 ml SF-900 medium, and the DNA mixtures were added to the cells. The cultures were allowed to incubate for 5 hours at 27°C. Following the 5 hr incubation period, the transfection mixture was removed and the cultures were replenished with 3 ml per well IPL-41 medium (Life Technologies) supplemented with 10% fetal bovine serum. Following a three day incubation period, the cells were harvested, centrifuged, and the supernatant that contained recombinant virus (designated as passage 1 or P1 stock) was removed and stored at 4°C. A larger viral stock was made by infecting 100 ml fresh Sf9 cells at 5 x 10^5 cells per ml with 0.5 ml of the P1 medium. This larger (P2) stock was then titered using a plaque assay protocol (O'Reilly et al., 1992), and used for production of the 11 alpha hydroxylase or oxidoreductase enzymes, separately or in combination with each other.

Figure 14 sets forth an immunoblot illustrating expression of *Aspergillus ochraceus* P450 11 alpha hydroxylase in baculovirus-infected insect cells harvested at 25 and 48 hours post infection. The nitrocellulose membrane was probed with a 1:1 mixture of antibodies prepared two rabbits immunized with a conjugated synthetic peptide 11aOH peptide 2 (SEQ ID NO 24).

Figure 15 sets forth an immunoblot illustrating expression of *Aspergillus ochraceus* P450 oxidoreductase in baculovirus-infected insect cells harvested at 25 and 48 hours post infection. The nitrocellulose membrane was probed with a 1:1 mixture of antibodies prepared two rabbits immunized with a conjugated synthetic peptide oxr peptide 1 (SEQ ID NO 26).

Example 17: Co-infection baculoviruses expressing of *Aspergillus ochraceus* 11 alpha hydroxylase and human oxidoreductase

Sf9 cells were co-infected with virus particles that contained the steroid 11 alpha hydroxylase cDNA and a separate virus containing a human NADPH P450-oxidoreductase. Both viruses were added at a multiplicity of infection (MOI) ratio of 0.1 : 0.01 (11 aOH to oxr). One day after infection, 0.9 µg/ml hemin chloride was added to the culture. The cells were harvested by centrifugation three days after

infection (unless specified differently), and the washed cell pellets were frozen until processed for sub-cellular fractions.

Example 18: Co-infection baculoviruses expressing of *Aspergillus ochraceus* 11 alpha hydroxylase and *Aspergillus ochraceus* oxidoreductase

Sf9 cells are co-infected with virus particles that contain the steroid 11 alpha hydroxylase cDNA and a separate virus containing *A. ochraceus* NADPH P450-oxidoreductase. Both viruses are added at a multiplicity of infection (MOI) ratio of 0.1 : 0.01 (11 aOH to oxr). One day after infection, 0.9 μg/ml hemin chloride is added to the culture. The cells are harvested by centrifugation three days after infection (unless specified differently), and the washed cell pellets are frozen until needed in subsequent experiments that require processing into for sub-cellular fractions.

Example 19: Preparation of subcellular fractions from baculovirus-infected insect cells

One half gram of the cell pastes from infected sf9 cells and uninfected control cells were thawed and suspended in 40 ml of 0.25 M sucrose with 10 mM KHPO4, adjusted to pH 7.4. The suspensions were homogenized using a Fisher Sonic Dismembrator, model 300 probe sonicator (Fisher Scientific, St. Louis, MO). The samples were transferred to a conical centrifuge tube (Corning Costar Corporation, Cambridge, MA) and subjected to centrifugation at 500 x g at 5°C for 15 minutes. The pellets were resuspended in the same volume of fresh buffer and viewed under a microscope to confirm complete lysis. Few or no whole cells were observed. The supernatants were then subjected to centrifugation at 10,000 x g for 30 minutes at 5°C to collect mitochondria, Golgi and other subcellular organelles. The pellets were resuspended in fresh buffer and subjected to centrifugation at 7,800 x g for 30 minutes at 5°C to collect mitochondria.

The mitochondrial pellets were resuspended in buffer as described about and the centrifugation was repeated. The mitochondrial pellets were resuspended in 2 ml buffered sucrose solution and stored at -80°C in 100 ul aliquots.

The supernatants from the original mitochondrial fractionation were subjected to centrifugation at 200,000 x g for 1 hour at 5°C. The microsomal pellets were resuspended in 2 ml buffered sucrose solution and stored at -80°C in 100 ul aliquots.

Microsomal Incubations

Incubation mixtures consisted of Sf9 microsomes (1.0 mg of protein/mL final concentration), an NADPH-generating system and 250 uM substrate (AD) in 100 mM potassium phosphate buffer, pH 7.4 or 150 mM HEPES buffer, pH 7.4. The NADPH-generating system was composed of the following at the indicated final concentrations: $MgCl_2$ (7.5 mM), D-glucose-6-phosphate (7.5 mM), NADP (0.80 mM), and glucose-6-phosphate dehydrogenase (1.0 units/mL). Incubations were carried out for the indicated times at 37°C in a water bath. Following incubation, reactions were terminated by the addition of 0.3 ml methanol. The samples were vortexed three times for two seconds and placed on ice, or stored at -70°C for later analysis.

Example 20: HPLC assays to measure conversion of steroid substrates to their hydroxylated counterparts

High Performance Liquid Chromatography (HPLC)

The HPLC method used to separate hydroxylated steroid compounds from steroid substrates, such as 11α-hydroxyandrostenedione from androstenedione, is a modified version of the testosterone hydroxylase assay, described by Sonderfan et al., *Arch. Biochem. Biophys.* **255**: 27-41, 1987). The standards for androstenedione and 11-beta-hydroxyandrostenedione were obtained from Sigma. 11-alpha-hydroxyandrostenedione (89.5% pure, with the major impurity being androstenedione) was provided by Searle Medicinal Chemistry. HPLC grade water and methanol were obtained from Burdick & Jackson.

The HPLC system consisted of a Model 1050 series pump, autoinjector and variable wavelength detector (Hewlett-Packard, Naperville, IL), and a Model TC-50 temperature controller and Model CH-30 column heater (both Eppendorf, Madison, WI).

Cell membrane fractions derived from insect cells transfected with recombinant baculoviruses expressing 11-hydroxylase and complementary electron transport proteins were analyzed for 11-hydroxylase activity in a reaction mixture containing 80 mM phosphate buffer, pH 7.4, 8 mM $MgCl_2$, and 0.9 mM $NADP^+$ in a final volume of 200 ul. In order to insure an adequate source of reducing equivalents, an NADPH regenerating system was provided by adding glucose-6-phosphate dehydrogenase (1.5 U/ml) and 8 mM glucose-6-phosphate. Steroid substrate (e.g., androstenedione) was provided at a final concentration of 0.3 mM.

Reaction mixtures were incubated at 37°C for 30 min. The reactions were terminated by the addition of 200 ul methanol and then placed on ice. Samples were pelleted by centrifugation to remove precipitated protein.

On one occasion, the incubation was carried out in a volume of 0.5 ml in siliconized polypropylene 1.5 ml microcentrifuge tubes at 37°C for 120 minutes. The enzyme, prepared from microsomal or mitochondrial fractions, was added and the substrate added at a concentration of 250 μM (e.g., 25 mM methanol stock solution of AD). The cofactor buffer was 100 mM potassium phosphate, pH 7.4, 7.5 mM $MgCl_2$, 7.5 mM glucose-6-phosphate, 0.80 mM NADP, and 1.0 units/mL glucose-6-phosphate dehydrogenase. HPLC samples were prepared by terminating the 0.5 ml reaction mixture by addition of 0.3 ml methanol, vortexing three times for 2 seconds and storing on ice. The tubes were spun for 5 minutes at ~20,000 x g in a microcentrifuge and the samples transferred to autosampler vials and capped.

Steroid components present in reaction mixtures and media extract were separated and analyzed by reverse phase HPLC using a 250 mm x 4 mm Vydac analytical C-4 column. Chromatograms were developed using a solvent gradient from 40% to 100% methanol over a ten minute time period and holding at 100% methanol for 5 minutes before re-equilibration to initial conditions. The column effluent was monitored for UV absorbance at both 254 and 220 nm.

Androstenedione, testosterone and monohydroxylated androstenedione metabolites were resolved on a Nova-pak C18 column, 4 micron, 3.9 x 150 mm (Waters Chromatography, Milford, MA) equipped with a 0.22 micron Rheodyne precolumn filter at 40°C and 1.0 ml mobile phase/min. A stepped gradient was utilized with water as mobile phase solvent A and methanol as solvent B. The initial concentration of solvent B was 42% for 6 min. The percentage of B was increased linearly to 45% over 4 minutes and then held for 3 minutes. The percentage of B was then increased linearly to 80% over 10 minutes and held there for an additional 2 minutes for a total run time of 25 minutes. The ultraviolet detection wavelength was 247 nm and the injection volume was 200 ul.

Both the "mitochondria" sample and the "microsomal" sample produced peaks matching the HPLC retention time of the 11α-hydroxyandrostenedione standard, while other fractions did not. These "mitochondria" and "microsomal" peaks were 3.2 and 2.3%, respectively, of the total peak area quantitated at 247 nm. The 11α-hydroxyandrostenedione standard was also spiked into a blank microsomal incubation sample at a concentration of 5.0 μg/mL. The concentration

of the "mitochondria" and "microsomal" 11α-hydroxyandrostenedione peaks were 1.75 and 1.31 μg/mL, after correcting for the purity of the standard (89.5%). These concentrations represent 2.3 and 1.7% of substrate converted to 11α-hydroxyandrostenedione, using a substrate concentration of 250 μM.

Figure 16 sets forth an HPLC tracing illustrating the conversion of androstenedione (AD) to its 11 alpha hydroxy counterpart after incubating AD with subcellular fractions prepared from baculovirus-infected insect cells expressing *Aspergillus ochraceus* 11 alpha hydroxylase and human oxidoreductase.

Example 21: Recognition of *Aspergillus ochraceus* 11 alpha hydroxylase and *Aspergillus ochraceus* NADPH cytochrome p450 reductase by immunoblotting using polyclonal antibodies generated against synthetic peptides

Proteins from Sf9 cell lysates (obtained from uninfected and recombinant baculovirus-infected cells) were loaded onto lanes of a 10% gradient acrylamide mini gel (BioRad, Hercules, CA) at equal concentrations (10 μg per well). The proteins were separated by electrophoresis at 16 mAmps constant current for approximatley 1 hr in a Tris-glycine buffer containing 0.1% SDS (Sigma, St. Louis, MO). The proteins were transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) for 40 min at 70 mAmp constant current. Primary antibodies were diluted 1:10 (from stock concentrations of 0.34 mg/ml IgG for anti-11 alpha hydroxylase (antibodies GN-1187 and GN-1188 prepared from peptide 11aOH peptide 2 CRQILTPYIHKRKSLKGTTD (SEQ ID NO: 24)), and 0.26 mg/ml IgG for anti-oxred (antibodies GN-2023 and GN-12024 prepared from oxr peptide 1 CTYWAVAKDPYASAGPAMNG (SEQ ID NO: 26)) and used to probe the nitrocellulose membrane. The antigens were detected using anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody as recommended by the manufacturer (New England Biolabs, Beverly, MA). Chemilumiescence was detected using luminol and peroxide reagents (New England Biolabs, Beverly, MA) following the protocol provided by the vendor. Light emission was recorded using X-OMAT AR film (Eastman Kodak Company, Rochester, NY). Images were recorded using a Minolta Dimage V digital camera (Minolta Corporation, Ramsey, NJ).

Example 22: Characterization of the *Aspergillus ochraceus* genomic DNA encoding 11 alpha hydroxylase and oxidoreductase

The approaches described above can be used to facilitate the identification of genes encoding steroid hydroxylases and oxidoreductases within the genome of *Aspergillus ochraceus* and closely related microorganisms, including *Aspergillus niger* and *Aspergillus nidulans*. Other preferred organisms are *Rhizopus oryzae, Rhizopus stolonifer, Streptomyces fradiae, Bacillus megaterium, Pseudomonas cruciviae, Trichothecium roseum, Fusarium oxysporum f. sp. cepae, Rhizopus arrhizus, and Monosporium olivaceum*. Other preferred organisms that are known to have steroid 11 alpha hydroxylase activity are described in the detailed description of the invention, above.

Briefly, genomic DNA is prepared and shotgun cloned into low copy artificial chromosomes propagated in bacteria. A large number of clones are sequenced to ensure statistical representation of the entire genome, and the sequences of overlapping clones merged to produce the final map and sequence of the genome. Analysis of the open reading frames, will reveal regions which are homologous to the steroid hydroxylase and oxidoreductase genes of the present invention, and regions of the translated open reading frames which are homologous to these enzymes using programs designed to facilitate multiple sequence alignments of nucleotide and protein sequence data such as BLAST, CLUSTAL W, and BoxShade. Genes which encode these proteins are obtained from the artificial chromosomes and recloned into expression vectors such as pFastBac1, transformed into appropriate host cells, which are assayed for the presence of enzymes capable of carrying out the conversion of steroid substrates to their oxidized counterparts.

It is intended that the scope of the present invention be determined by reference to the appended claims. It is recognized that a number of variations can be made to this invention as it is currently described but which do not depart from the scope and spirit of the invention without compromising any of its advantages. These include isolation of homologous genes from microorganisms known to carry out 11 alpha hydroxylation of steroid substrates, preferably fungi and bacteria. This invention is also directed to any substitution of analogous components. This includes, but is not restricted to use of these techniques to isolate other P450s which are involved in steroidogenesis, including hydroxylases that act at other

positions in the core molecule, and use of these enzymes to facilitate bioconversion of steroid intermediates in modified host microorganisms.

All references, patents, or applications cited herein are incorporated by reference in their entirety, as if written herein.

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